# Twelve-Month Inhalation Study on

# Room-Aged Cigarette Sidestream Smoke in Rats

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room-aged sidestream smoke inhalation

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The present study extends the current scope of rat inhalation studies on surrogates of environmental tobacco smoke. The 12-month inhalation period enabled an investigation of the potential progression or occurrence of new morphologic effects from subchronic to chronic inhalation. In addition, pulmonary inflammation and oxidative DNA damage were investigated. Female Wistar rats were whole-body exposed to room-aged cigarette sidestream smoke (RASS) generated from the reference cigarette 1R4F at 6 and 12 µg total particulate matter/l for 12 h/day, 5 days/week, and 12 months. To enable an evaluation of the exposure mode, another group of rats was exposed head-only to 12 µg total particulate matter/l for 7 h/day. Whole-body exposure conditions per se resulted in changes of the RASS composition. An analysis of urinary nicotine metabolites showed that with whole-body exposure, RASS components, such as nicotine, were additionally taken up by routes other than inhalation. Independent from the exposure mode, blood carboxyhemoglobin and the hemoglobin adduct of 4-aminobiphenyl were used as biomarkers for the RASS concentration and dose, respectively. Histopathological changes were minimal to moderate reserve cell hyperplasia and slight squamous metaplasia of the respiratory epithelium as well as minimal reserve cell hyperplasia and atrophy of the olfactory epithelium in the anterior nasal cavity; slight eosinophilic globules in sustentacular cells of the olfactory epithelium in the anterior and posterior nasal cavity; pronounced squamous metaplasia and hyperplasia in the larynx at the base of epiglottis; and slight reserve cell hyperplasia in the bronchial respiratory epithelium. Most of the changes were adaptive and similar in type and degree to those seen in previous subchronic RASS inhalation studies. A flow cytometric analysis of bronchoalveolar lavage cells, i.e., alveolar macrophages, lymphocytes, and polymorphonuclear leukocytes, did not show signs of pulmonary inflammation after 6 or 12 months of inhalation. As a measure for oxidative DNA modifications, 8-hydroxy-deoxyguanosine was determined in the lungs and nasal epithelia. No change was seen for this parameter at either time point in the lungs. There

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*was a slight but not consistent increase in the nasal respiratory and olfactory epithelia as well as in urinary 8-hydroxy-deoxyguanosine excretion. In summary, there was little indication for progression or occurrence of new effects from 3 or 6 months to 12 months of RASS inhalation. There were also no signs of inflammation or oxidative DNA modification in the lungs. Chronic head-only exposure to RASS was shown to be technically feasible and is generally considered preferable for smoke inhalation studies over whole-body exposure to avoid artificial changes in smoke composition and the non-inhalative uptake of smoke constituents.*

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Environmental tobacco smoke (ETS) was classified as a class A (known human) carcinogen by the US Environmental Protection Agency (US EPA, 1992). The agency based this decision on the analogy to mainstream cigarette smoke (MS) as well as on the results of epidemiological studies. It acknowledged that lifetime animal inhalation studies were lacking, which implies that further experimental toxicology is necessary to evaluate the claimed biological plausibility of US EPA's classification.

Since this classification, three long-term inhalation studies on A/J mice were reported using different sidestream smoke (SS) surrogates for ETS (Witschi et al., 1995a, 1997a,b). The first study, six months of exposure to relatively fresh SS at a concentration of 4 µg total particulate matter (TPM)/l, did not show a difference in the rate of lung tumors compared to controls. In the second and third study, five months of exposure to a mixture of SS and MS at concentrations up to 87 µg TPM/l plus a 4-month postinhalation period, increased rates of pulmonary adenomas in the exposed mice were reported. However, apart from the highly toxic dose levels used, there were several inconsistencies in the latter studies, such as the large variation in the spontaneous lung tumor rate and the lack of smoke exposure-related non-neoplastic lesions in the lungs, which leave some questions open about the relevance of this experimental design. For rats, no long-term SS inhalation study has been reported to date although the rat is the most frequently used species in subchronic inhalation studies (Witschi et al., 1995b).

The present chronic study extends the current scope of rat inhalation studies on ETS surrogates. With an inhalation period of 12 months, an investigation of the potential progression of respiratory tract histopathological changes or occurrence of new changes from subchronic to chronic inhalation is made possible. In addition, the investigation of mechanistic end points has been included in this study in line with EPA's proposed guidelines for risk

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assessment (US EPA, 1996) to obtain information on potential long-term/tumorigenic effects of SS using this experimental design. Pulmonary inflammation was assessed by investigating the composition of bronchoalveolar lavage cells, while oxidative DNA damage was assessed by determining tissue and urinary 8-hydroxy-deoxyguanosine (8-OHdG) levels. Other end points, such as cytokeratin expression, an epithelial cell differentiation marker, were also investigated and will be reported elsewhere (*cf.*, Schlage et al., 1997). The results of this study should help in the design and interpretation of possible long-term bioassays with regard to exposure levels, exposure mode, and/or mechanisms involved in chronic toxicity and possibly carcinogenicity, and thus contribute to a quantitative risk assessment of ETS (*cf.*, Witschi et al., 1995b).

Since ETS can not be reproducibly generated as required for chronic laboratory experiments, ETS surrogates have been developed for laboratory studies, e.g., aged and diluted SS (Coggins et al. 1993; Ji et al., 1994) and, more recently, room-aged SS (RASS) (Voncken et al., 1994; Haussmann et al., 1998). Although the carbon monoxide (CO) concentration - as a proxy for the number of cigarettes smoked per air volume - may be the most relevant basis to evaluate experimental SS-related effects, the TPM concentration was used in the present study to enable a comparison with environmental or other experimental SS studies. Concentrations in the range used in the present study (6 and 12 µg TPM/l) have shown to be effective in subchronic studies on rodents in producing a spectrum of histopathological (von Meyerinck et al. 1989), biochemical (Ji et al., 1994), genotoxic (Lee et al., 1993), and cell proliferative changes (Witschi et al., 1995a) and were, therefore, considered suitable for a chronic RASS inhalation study. The RASS concentrations employed in this study were approximately 100-fold higher than the maximum of the average concentrations of respiratory suspended particles (RSP) reported for ETS (Guerin et al., 1992; US EPA, 1992; Jenkins et al., 1996).

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The aforementioned long-term SS inhalation studies (Witschi et al., 1995a, 1997a,b) as well as some subchronic SS inhalation studies (e.g., von Meyerinck et al., 1989) were conducted using the whole-body exposure mode. This is most probably related to the convenience for both experimental animals and staff. However, when using an aerosol such as cigarette smoke, this exposure mode might be subject to confounding influences, such as oral uptake during grooming. Differences between the whole-body and head-only exposure modes were reported for the uptake of smoke constituents (Mauderly et al., 1989; Chen et al., 1995). Similarly, pronounced differences in the uptake and toxicity of test atmospheres other than cigarette smoke were observed between whole-body and head-only exposure modes (Langard and Nordhagen, 1980; Wolff et al., 1982; Iwasaki et al., 1988). In the present study, therefore, the head-only exposure mode was compared to the whole-body mode with regard to various biomarkers of exposure and effect. The test substance uptake was maximized by extending the daily exposure duration as long as feasible for each exposure mode.

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## MATERIALS AND METHODS

### Experimental Design

Female rats were whole-body exposed to RASS at concentrations of 6 and 12 µg TPM/l (WB6 and WB12) or to filtered, conditioned fresh air (WB0, sham-exposed group) for 12 h per day, 5 days per week, for 12 months (Table 1). For the comparison of exposure modes, another group of rats was head-only exposed for 5 days per week for 12 months to the high RASS concentration (HO12), but only for 7 h per day, which is considered the maximum daily exposure duration for this exposure mode. Due to laboratory capacity restraints, the inclusion of a head-only sham-exposed control group was not possible. However, this is not considered to seriously impact the comparison of the two exposure modes for most of the end points investigated. The two exposure modes were compared based on the TPM concentration as well as on the daily TPM dose for those end points presumably unaffected by tube restraint. Interim investigations of pulmonary inflammation and oxidative DNA damage, end points not previously included in subchronic SS inhalation studies, were performed after 6 months of inhalation for WB0 and WB12 only.

The study was performed in conformity with the American Association for Laboratory Animal Science Policy on the Humane Care and Use of Laboratory Animals (1991).

### Experimental Animals

Female outbred Wistar rats (Crl: (WI)WU BR), bred under specified pathogen-free conditions, were obtained from Charles River (Sulzfeld, Germany). Wistar rats were used in the present study because of their potential suitability for long-term inhalation bioassays in terms of longevity, moderate body weight development, low rate of spontaneous tumors, particularly in the lungs, and sensitivity to rat respiratory tract carcinogens (Woutersen et al.,

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1986; Kroes et al, 1988, Gupta et al., 1990; Vandenberghe, 1990; Bomhard and Rinke, 1994; Heinrich et al., 1995; Sanders and Lundgren, 1995). Female rats were used, because they showed a higher sensitivity in several long-term aerosol inhalation studies (Brightwell et al., 1989; Nikula et al., 1995), while in subchronic SS inhalation studies no difference was reported for the histopathological changes between male and female rats (Coggins et al., 1992). Wistar rats have not been used for the evaluation of SS-induced histopathological changes in the respiratory tract to date.

The respiratory tracts of 5 randomly selected rats were histopathologically examined on arrival; no abnormal findings were observed. Serological screening of 10 to 15 rats performed on arrival and after 6 and 12 months of inhalation did not detect antibodies to rat-related viruses, such as hantavirus, lymphocytic choriomeningitis virus, murine adenovirus, parvovirus H-1, pneumonia virus of mice, rat coronavirus/sialodacryoadenitis virus, rat virus, reovirus 3, rodent orphan parvovirus, Sendai virus, and Theiler's murine encephalomyelitis virus, to the bacteria *Clostridium piliforme*, *Mycoplasma pulmonis*, and cilia-associated respiratory bacillus, nor to the protozoon *Encephalitozoon cuniculi*.

The rats were individually identified using subcutaneous transponders (IMI-1000, Plexx, Elst, Netherlands; data acquisition by DAS-4001, Uno, Zevenaar, Netherlands). Following a 7-day acclimatization period before exposure, they were randomly allocated to the two RASS groups and the sham exposure group for whole body exposure (WB0 and WB12: 96 rats/group; WB6: 48 rats) as well as to the high RASS concentration group for head-only exposure (HO12: 48 rats). The age of the rats at the start of the inhalation period was between 55 and 70 days. The mean body weight at that time was 110 g (SD: 10 g).

The rats were barrier maintained in an animal laboratory unit with controlled hygienic conditions. The laboratory air (filtered, fresh air) was conditioned. Positive pressure was

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maintained inside the laboratory unit. Room temperature and relative humidity were maintained at 22 °C (SD: 1 °C) and 61 % (SD: 9 %), respectively. The light/dark cycle was 14 h/10 h. The whole-body exposed rats were exposed and housed in wire mesh cages (2 rats/cage), whereas the head-only exposed rats were housed between exposures in transparent polycarbonate cages (2 rats/cage) on sterilized softwood granulate (Braun & Co., Battenberg, Germany). A sterilized, fortified pellet diet (MRH FF, Eggersmann, Rinteln, Germany) from cage lid racks and sterilized water from bottles with sterilized sipper tubes were supplied ad libitum in each cage. Food was not available to the rats during the daily exposure periods. During exposure, drinking water was not available to the head-only exposed rats. Chemical analyses of food, water, and bedding material confirmed compliance with the requirements set forth by the National Toxicology Program (1991). Good hygienic conditions within the animal housing and exposure rooms were maintained as evidenced by the results of the bacteriological examinations of the laboratory surfaces and air as well as of the rat diet and drinking water.

**RASS Generation**

The University of Kentucky reference cigarette 1R4F (MS yields per cigarette: 10.8 mg TPM, 0.80 mg nicotine, and 11.6 mg CO; Tobacco and Health Research Institute, 1990) was used for SS generation as previously described for another reference cigarette (Haussmann et al., 1998). The cigarettes were smoked in basic accordance with the International Organization for Standardization as generally applied to MS generation. Room-aging was performed by continuously passing diluted SS at a rate of 56 $m^3/h$ through a 28-$m^3$ experimental aging room with non-inert surfaces, resulting in RASS of a mean age of 0.5 h. In the aging room were materials usually found in residences and/or offices, such as wallpaper painted with a latex-based white paint (29 $m^2$), vinyl floor tiles (11 $m^2$), and a polycarbonate

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window (2 $m^2$) (*cf.*, Voncken et al., 1994). The materials in the aging room were unexposed at the start of the inhalation. The painted wallpaper was replaced every 13 weeks. A ceiling fan was operated to facilitate uniform distribution of the RASS. The room was illuminated by fluorescent "daylight" lamps (Lumilux L58W/11, Osram, Munich, Germany). Two heat exchangers (approximately 60 $m^2$ surface area) were used to keep the room temperature constant. RASS was conveyed via glass tubing to the exposure chambers. RASS generation was started approximately 2.5 h before the start of the daily exposure to achieve a steady-state test atmosphere for inhalation. During overnight, non-smoking periods, the room was flushed with filtered, conditioned fresh air at 56 $m^3$/h.

The test atmosphere for WB6 was obtained by diluting the RASS from the aging room with filtered, conditioned fresh air. Whole-body exposure normally results in slight losses of TPM, mainly due to particle deposition in the chamber and on fur. In order to obtain the same TPM concentration in WB12 and HO12, the RASS from the aging room was diluted by 20 % with filtered, conditioned fresh air before entering the head-only exposure chamber.

Sham-exposed rats (WB0) were exposed to filtered, conditioned fresh air under the same conditions as the whole-body RASS-exposed rats.

**Analytical Characterization of the Test Atmospheres**

At designated time intervals, a broad range of analytes was determined to characterize the test atmospheres, to evaluate the reproducibility of the test atmosphere generation, and to detect possible cross contamination in the sham-exposed group. Samples were collected within the exposure chambers at sites representative for the breathing zone of the rats. CO was continuously monitored. TPM was determined at least once per day. The other analytes were determined at less frequent intervals from weekly to twice a year.

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The analytical methods used to determine TPM, CO, nicotine, nitrogen oxides, aldehydes, ammonia, and the particle size distribution as well as those for temperature and relative humidity in the exposure chambers were performed as previously described (Haussmann et al., 1998). The other analytes were determined as follows: 3-Ethenyl-pyridine and nicotine were determined in parallel using 2-ethenyl-pyridine as internal standard. Solanesol was determined in particulate matter after trapping on Fluoropore membrane filters (pore size: 1 µm; Millipore, Eschborn, Germany) by reversed phase HPLC (Lichrospher RP-select B, 5 µm, 125 x 3 mm, Merck, Darmstadt, Germany) and UV detection (HP 1090, Hewlett Packard, Waldbronn, Germany). Isoprene, toluene, 1,3-butadiene, and benzene were trapped in methanol at -78 °C and determined by gas chromatography/mass spectrometry (GC/MS; Hewlett Packard 5890A/5970B) with a DB-5.625 column (30 m x 0.25 mm, J and W, Fisons, Wiesbaden, Germany). Phenols were determined in the particulate phase after trapping on Cambridge type glass fiber filters (Gelman, Ann Arbor, MI, USA), extraction, and silylation using GC/MS with a DB-5.625 column. Polycyclic aromatic hydrocarbons were extracted from a TPM-loaded glass fiber filter with methanol/water followed by back extraction with hexane, clean-up by solid phase extraction (Bakerbond amino, Baker, Gross-Gerau, Germany), and analyzed by GC/MS with a DB-17 column (30 m x 0.25 mm, J and W). N-nitrosamines were trapped in citrate/phosphate buffer with ascorbic acid and on glass fiber filters connected in series. The combined dichloromethane extracts were washed with a sodium hydroxide solution and cleaned by adsorption chromatography on aluminum oxide. The N-nitrosamines were determined by GC with a DB-5 column 30 m x 0.53 mm (ICT, Bad Homburg, Germany) and a thermal energy analyzer (TEA 543, Thermo Electron Corporation, via Isconlab, Heidelberg, Germany). For the determination of the metals, SS was collected on membrane filters with 0.22 µm pore size (GSWP 02500, Millipore). After the filters were digested with

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nitric acid, the metals were determined by graphite furnace atomic absorption spectrometry by Henkel KGaA (Düsseldorf, Germany).

**Animal Exposure System**

The whole-body exposure chambers made of glass and stainless steel were equipped with 24 stainless steel wire mesh cages, which were mounted above stainless steel excretion pans. Each cage had a separate supply of test atmosphere. The flow rate through the chambers was 180 l/min. The position of the cages within the chamber was systematically changed on a weekly basis. Rats were exposed to RASS as well as to filtered, conditioned fresh air (sham-exposed control) for 12 h per day, 5 days per week, using two exposure chambers per group for WB0 and WB12 and 1 chamber for WB6.

Another group of rats (HO12) was head-only exposed to RASS for 7 h per day, 5 days per week. This exposure system was described previously (Haussmann et al., 1998). The position of the rats in the chamber was systematically changed on a daily basis.

**In-Life Observations**

The rats were observed daily for mortality, moribundity, signs of overt toxicity, or injuries. Detailed checks on general condition and behavior of the rats were performed on 3 rats/group per day shortly after the end of the daily exposure throughout the first 3 months, and two times per week in months 4 to 6. Starting with month 7, tumor checks were performed two times per week on all rats. The body weight of the individual rats was determined one day after their arrival, at the start of the inhalation period, and once per week during the inhalation period.

**Biomonitoring**

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In order to provide an estimate of the amount of test atmosphere taken up by the rats, respiratory frequency and tidal volume were determined on at least 10 rats/group by whole-body plethysmography, three times during the inhalation period, as previously described (Haussmann et al., 1998). Rats were allowed to adapt to the plethysmographic tubes prior to data acquisition. During the adaptation and acquisition periods, they were continuously exposed to the test atmospheres.

To monitor exposure to CO, steady-state proportions of blood carboxyhemoglobin (HbCO) were determined in 5 rats/group according to Klimisch et al. (1974), three times during the inhalation period. The blood samples were collected after at least 5 h of exposure, a duration sufficient to obtain steady-state HbCO proportions (*cf.*, Tyuma et al., 1981). Rats were removed from the exposure chambers for a short period of time, and blood samples were taken under diethyl ether narcosis by puncturing the retro-orbital sinus with glass micropipettes.

To monitor exposure to aromatic amines, hemoglobin adducts of nine aromatic amines were determined in 7 to 8 rats/group following 12 months of RASS inhalation according to Kutzer et al. (1997). The hydrolyzed amines were derivatized with pentafluoropropionic anhydride (Aldrich, Steinheim, Germany) and analyzed using gas chromatography/mass spectrometry with negative chemical ionization in the SIM mode (DB-5MS column, J&W Scientific, Folsom, CA / TSQ 700, Finnigan, Bremen, Germany). For internal standardization, $D_5$-aniline (Aldrich) and $D_9$-2-aminobiphenyl (IC Chemikalien, Ismaning, Germany) were used.

To provide an estimate of the amount of nicotine taken up by the rats, nicotine metabolites were determined in urine collected from 6 rats/group over 24 h, three times during the inhalation period. During the 7-h head-only exposure period, the urine was collected using specially modified exposure tubes. During the postexposure period and for the whole-body

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exposed groups, custom-made metabolism cages were used. All samples per rat and time point were combined, centrifuged, and stored frozen. The nicotine metabolites were determined by HPLC after derivatization with 1,3-diethyl-2-thiobarbituric acid (Rustemeier et al., 1993). The separation of all analytes in one chromatographic run at ambient temperature was enabled by modifying the derivatization conditions (0 °C and pH 3.3), the composition and pH of solvent A (54 mM 1-pentanesulphonic acid, 5 mM 1-heptanesulphonic acid, pH 5.0), and the solvent and flow program.

**Gross Pathology and Organ Weight**

Necropsy was performed without prior fasting. On the day following the last exposure at the end of the 12-month inhalation period, 8 rats/group were killed and examined as previously described (Haussmann et al., 1998). The weights of the lungs with larynx and trachea, liver, heart, adrenal glands, and kidneys were determined.

**Histopathology**

Histopathology of the respiratory tract was performed after 12 months of inhalation as previously described (Haussmann et al., 1998) with additional levels in the posterior nasal cavity (levels 3 and 4 according to Young, 1981). Processing and sectioning of the samples was performed at Huntingdon Research Centre (Huntingdon, Cambridgeshire, U.K.). The larynges of most of the rats were not reproducibly sectioned at the pre-defined levels needed for a semi-quantitative evaluation and a comparison to previous data. Thus, they could only be evaluated qualitatively. All slides were read by a veterinary pathologist in a blinded manner with experience in cigarette smoke-related changes in the respiratory tract of rodents. All pathological findings were scored according to a defined severity scale from 0 to 5 (marked effects). Mean severity scores were calculated based on all rats of a group.

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**Composition of Bronchoalveolar Lavage Cells**

As a measure for possible inflammatory processes in the lungs, a differential count of bronchoalveolar lavage cells was performed using flow cytometry. For this purpose, 8 rats/group were killed as described following 6 (WB0 and WB12 only) and 12 months (all groups) of RASS inhalation. The lungs were lavaged via the trachea with 120 ml lavage medium per rat in 10 lavage cycles. The lavage medium was Dulbecco's phosphate buffered saline without calcium and magnesium (Biochrom, Berlin, Germany) supplemented with 3.25 g/l bovine serum albumin (Sigma) (pH 7.2). The viability of the lavaged cells of all groups was 95.5 % (SE: 0.4 %) as determined by the Trypan blue dye exclusion method. After fixation of the cells, they were incubated on ice for 48 h in a cell membrane permeabilization medium (according to Sander et al., 1991, with some modifications) to enable labeling of an intracellular epitope with the *pan* rat macrophage antibody ED1-fluoresceinisothiocyanate (FITC) conjugate (15 mg/l; Serotec, Kidlington, UK). Propidium iodide was added as a nucleic acid marker at 5 mg/l. The cells were analyzed using a Cytofluorograf 50H flow cytometer (Ortho Diagnostic Systems, Westwood, MA) in conjunction with the Cicero data acquisition and analysis workstation (Cytomation, Ft. Collins, CO). The cell populations (alveolar macrophages (AM), lymphocytes, and polymorphonuclear leukocytes (PMNL)) were identified by their characteristic appearance on a dot plot histogram of green (ED1-FITC; 515 - 530 nm) *vs.* red (nucleic acid content; ≥ 630 nm) fluorescence. From each sample, 30,000 counts were collected. Microscopic evaluation of Pappenheim-stained (Romeis, 1968) smears of bronchoalveolar lavage cells confirmed the flow cytometric results.

An aliquot of the above fixed lavaged cells was analyzed for spontaneous fluorescence. The system was internally calibrated by adding monodisperse (diameter: 1.95 µm) fluorescent latex beads (Duke Scientific, Palo Alto, CA) into the cell suspension. After gating the AM

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population from a dot plot histogram of axial light loss *vs.* right angle light scatter, the green fluorescence (515 - 530 nm) was quantified in arbitrary units normalized to that of the admixed latex beads. From each sample, 15,000 counts were collected.

**8-Hydroxy-Deoxyguanosine Determination**

As a measure for possible oxidative DNA damage, 8-OHdG was determined in 8 rats/group after 6 (WB0 and WB12 only) and 12 months (all groups) of inhalation. During the last exposure day, rats were killed as described. The lungs were perfused in situ with isotonic saline to remove erythrocytes. The lungs as well as the nasal respiratory epithelium and the nasal olfactory epithelium were stored frozen at -70 °C until further processing. DNA extraction was performed according to Gupta (1984) with emphasis on fast tissue processing to prevent both DNA repair and artificial DNA oxidation. Enzymatic digestion of DNA as well as chromatographic separation (Nucleosil 100-5 C18 precolumn, CS Chromatographie Service, Langerwehe, Germany; Novapack C18, Waters Millipore; HPLC HP 1050, Hewlett Packard) and electrochemical determination (HP 1049A, Hewlett Packard) of 8-OHdG were performed according to Shigenaga et al. (1990). The amount of deoxyguanosine (dG) present in the samples was determined by its absorbance at 245 nm.

As an integrative monitor for oxidative DNA damage without specification of the organs involved, 8-OHdG excretion in urine was determined in 5 to 6 rats/group at 5 and 12 months of inhalation. Twenty-four hour urine samples were collected as described for nicotine biomonitoring. The purification of 8-OHdG from the rat urine was performed according to Shigenaga et al. (1990) with some modifications. An internal standardization method was employed using $^{3}$H-labeled 8-OHdG prepared from [1',2'-$^{3}$H]-dGTP (Amersham, Braunschweig, Germany) according to Shigenaga et al. (1990). Interfering substances in the

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eluate were oxidized at +0.35 V before the actual 8-OHdG determination using an additional electrochemical detector.

### Statistical Analysis

The following statistical tests were performed on the biological data: For the overall comparison of the whole-body RASS- and sham-exposed groups (WB0, WB6, and WB12), the one-way analysis of variance for continuous data (Sachs, 1982) and the generalized Cochran-Mantel-Haenszel test (Koch and Edwards, 1988) for ordinal data were used with the TPM concentration as the stratifying variable. If the overall comparison showed a significant difference, the Duncan test (Duncan, 1955) and the generalized Cochran-Mantel-Haenszel test were applied to continuous and ordinal data, respectively, for a pairwise comparison between the groups. All tests were conducted at the nominal level of significance of $\alpha = 0.05$ (2-tailed). Due to the large number of parameters analyzed, no correction for multiple testing was applied, which would have made the tests very insensitive. Statistical significances, therefore, have to be considered as explorative indicators rather than confirmatory evidence.

## RESULTS

### RASS Composition

Throughout the 12-month inhalation period, RASS was reproducibly generated and delivered to both types of exposure chambers. The analytical characterization of the sham and RASS exposure groups is shown in Table 2. As targeted, the TPM concentrations determined in the high concentration groups were the same for both exposure modes. The concentrations of most of the particulate phase constituents paralleled the TPM concentrations in the various groups. However, the catechol concentration in HO12 was almost twice as high as in WB12, for which there is no explanation. To achieve equal TPM

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concentrations in the high concentration groups, the TPM losses in the whole-body chambers were compensated by dilution of the RASS entering the head-only chamber. This was reflected by a 20 % lower CO concentration in HO12 compared to WB12. Most of the other gas phase components paralleled CO, with the exception of nicotine and formaldehyde, which were 30 and 70 % lower, respectively, in WB12 than expected based on the HO12 data if a proportional dilution of all RASS components would be assumed. The carbon dioxide concentration was determined both at the inlet and outlet of the exposure chambers to assess its concentration in RASS as well as the concentration added by the exhalate of the rats, respectively.

The particle size distribution was the same for all RASS exposure groups regardless of the exposure mode, with an average median mass aerodynamic diameter of 0.42 µm and a geometrical standard deviation of 1.8.

The relative humidity in the sham-exposed group was 58 ± 8 % (mean ± SD); this is considered to be representative for the other exposure groups. The temperature within the exposure chambers were between 22 and 24 ºC (SD: 1ºC). These environmental conditions complied with the exposure conditions specified by the OECD (1981).

**In-Life Observations**

There was no RASS-related mortality. Shortly after the end of the daily exposure, detailed checks of the rats revealed findings that occurred more often in rats in one or both high RASS concentration groups than in those of the sham-exposed group, *i.e.*, secretion from the Harderian glands, slight yellow-brown discoloration of the fur, and impaired gripping ability. No other RASS-associated effects on the general condition and behavior of the rats were detected.

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Signs of eye inflammation were found starting at 7 months peaking at 9 months of inhalation and were predominantly found in WB0 and HO12 (40 to 50 % of all rats) and less frequently in WB6 and WB12 (approximately 10 %). A cross-check of the data revealed no influence of this local inflammation on the other parameters of the study.

**Biomonitoring**

**RASS Uptake** Statistically significant decreases in respiratory frequency were seen in the whole-body exposed rats accompanied by a tendency to increased tidal volume resulting in slight decreases in the minute volume. For example, after 5 months of inhalation, the minute volume was 180 ± 7, 168 ± 8, and 162 ± 7 ml (mean ± SE) for WB0, WB6, and WB12, respectively. The respiratory minute volume for HO12 rats was 130 ± 6 ml. Daily RASS TPM doses were calculated taking into account these differences in respiratory minute volume, body weight, TPM concentrations, and daily exposure durations, the results being 2.8, 6.0, and 3.1 mg/kg body weight for WB6, WB12, and HO12, respectively. Thus, apart from the conventional comparison based on equivalent TPM concentrations (WB12 *vs.* HO12), a further comparison based on equivalent daily TPM doses is possible between WB6 and HO12.

**Carboxyhemoglobin** The steady-state HbCO proportions were in agreement with those expected based on the CO concentrations in the various test atmospheres (Figure 1A).

**Hemoglobin Adducts** Following 12 months of inhalation, a TPM dose dependent increase in hemoglobin adducts of 4-aminobiphenyl (4-AB) was found for WB0, WB6 / HO12, and WB12 (Figure 1B). The adduct levels for the other aromatic amines investigated (aniline, *o*-, *m*-, *p*-toluidine, 2-ethylaniline, 2,4-dimethylaniline, *o*-anisidine, and 3-aminobiphenyl) were

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not statistically significantly different among the groups, although a notable increase (up to 6-fold) in the 3-aminobiphenyl adduct level was seen (data not shown).

**Nicotine Uptake** For the evaluation of the nicotine uptake, five major urinary nicotine metabolites were determined, *i.e.*, nicotine-N'-oxide, nornicotine, cotinine, *trans*-3'-hydroxycotinine, and norcotinine. Nicotine itself was also determined but not used in this evaluation, since control experiments showed that aerosol nicotine directly dissolved in the urine collected in the whole-body chambers. The sum of the five metabolites excreted over 24 h at 5 months of RASS inhalation is presented in Figure 2A. Similar data were obtained at 12 months of inhalation. At 3 weeks of inhalation, the total amount of metabolites excreted in the whole-body exposed groups was up to 50 % lower than at the later time points. The amount of nicotine metabolites excreted, and correspondingly the nicotine uptake, increased dose dependently in the whole-body exposed groups to levels which far exceeded that in the head-only exposed rats. This is partially due to the longer daily exposure duration and the higher respiratory minute volume for the rats in the WB groups: A theoretical uptake of inhaled nicotine was calculated using the nicotine concentrations in the test atmospheres, the exposure durations, the respiratory minute volume, the latter being calculated based on the body weights of the rats at the respective time points (Guyton, 1947), and assuming total absorption. Based on this theoretical uptake, the nicotine uptake in the whole-body exposed rats was still 2- to 3-fold higher than that in the head-only exposed rats (Figure 2B). The sum of the five metabolites excreted in HO12 accounted for almost 50 % of the estimated nicotine taken up. Overall, the pattern seen for the five nicotine metabolites relative to each other was similar at all three time points as well as for the different dose levels and exposure modes (data not shown).

**Body Weight Development**

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The body weight of the rats increased throughout the inhalation period (Figure 3). After 3, 6, 9, and 12 months of inhalation, the body weight gain was statistically significantly lower by 5 to 8 % in WB12 compared to WB0; with the exception of the 12-month time point, the body weight gain in WB6 was statistically significantly lower by 4 to 6 % compared to WB0 (SE 1 to 2 % in all cases). The pronounced body weight effect seen in HO12 is considered to be mainly due to the exposure mode (Griffith and Standafer, 1985). A direct evaluation of the RASS effect on body weight development under head-only conditions was beyond the scope of the present study since a corresponding sham-exposed head-only control group had not been included.

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**Gross Pathology and Organ Weights**

No RASS-related gross pathological changes were observed. The only statistically significant change in absolute organ weights in the whole-body exposed groups was a slightly decreased absolute heart weight in WB12 (-9 ± 2 %), which was no longer apparent when normalized to the respective body weight. As for the body weights, possible organ weight effects in HO12 could not be evaluated due to the lack of a corresponding sham-exposed head-only control group.

**Histopathology**

In the anterior level of the nose (level 1), patchy, slight to moderate reserve cell hyperplasia of the respiratory epithelium was observed (Table 3). This effect was concentration dependent in the whole-body exposed rats and similar in degree and incidence in HO12 and WB12. Slight squamous metaplasia of the respiratory epithelium was observed in WB12, this effect being seen in only a few rats in WB6 and HO12. Minimal goblet cell hyperplasia was also seen. At level 2, minimal epithelial changes were seen in the RASS-exposed groups, such as reserve cell hyperplasia and atrophy of the olfactory epithelium and reserve cell hyperplasia of the respiratory epithelium. At levels 2 and 3, slight eosinophilic globules were observed in the sustentacular cells of the olfactory epithelium of the RASS-exposed groups. There were no other histopathological findings in levels 3 and 4 of the nasal cavity.

The few larynges that could be evaluated as intended showed distinct squamous metaplasia of the pseudostratified epithelium and hyperplasia of the squamous epithelium at the base of epiglottis (Table 3). Those laryngeal sections that were not cut at the pre-defined levels could only be qualitatively evaluated. This evaluation confirmed epithelial hyperplasia

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and squamous metaplasia to be the only findings observed in this organ. Further, the results given in Table 3 are representative for the findings in each of the respective groups. These effects were pronounced and seemed to be dose-dependent.

At the tracheal bifurcation, slight reserve cell hyperplasia of the respiratory epithelium was observed in only a few rats (not statistically significant; Table 3). In the pulmonary bronchial respiratory epithelium of the lungs, the degree of this finding was similar but the incidence was higher occurring most frequently in HO12. For both WB6 and WB12, this finding was statistically significantly different from WB0. The number of goblet cells was similar in all groups. A slight accumulation of pigmented AM was seen in WB12 and HO12.

The histopathological findings in the upper respiratory tract were less pronounced in HO12 compared to WB12. In the larynx at the base of epiglottis, the degree of the findings in HO12 was similar to that in WB6 (equal TPM doses). In the lower respiratory tract, the HO12 findings were closer to those seen in WB12, and the accumulation of pigmented AM was even seen at a higher incidence in HO12 than in WB12.

**Composition of Bronchoalveolar Lavage Cells**

There was a slight but statistically insignificant increase in the number of AM and thus of the total number of bronchoalveolar lavage cells with increasing TPM concentrations after 12 months of RASS inhalation (Table 4). No effect was seen for the number of lymphocytes and PMNL. Thus, no sign of a RASS-related inflammatory effect was observed. The same holds true for the comparison of WB12 and WB0 following 6 months of RASS inhalation (data not shown).

A green fluorescence was observed in the AM, the intensity of which increased with the RASS concentration (Table 5). For WB12, the intensity was similar following 6 or 12 months

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of RASS inhalation indicating that a saturation or a steady-state equilibrium was already obtained after the shorter inhalation period.

**8-Hydroxy-Deoxyguanosine Formation**

**Tissue Content** Both after 6 and 12 months of RASS inhalation, the nasal 8-OHdG content increased in WB12 compared to WB0 (Figure 4A and B); this increase was statistically significant in two out of four cases. The most pronounced effect was seen in the respiratory epithelium after 12 months of inhalation (+158 %). Overall, a steady-state equilibrium between formation and repair of this modification seemed to be reached already by the 6-month time point. The results for the head-only exposed group are difficult to interpret, because a tube restraint-related effect cannot be excluded.

In the lungs, the 8-OHdG content did not change statistically significantly. However, a consistent trend to lower 8-OHdG levels (up to 30 %) was observed at both time points regardless of the exposure mode (Figure 4C).

**Urinary Excretion** At 5 months of RASS inhalation, an increased excretion of 8-OHdG was observed in the whole-body exposed groups with increasing RASS concentrations (Figure 5). Such an effect was not seen at 12 months of inhalation. Again, a contribution of a tube restraint-related effect cannot be excluded for the HO12 results.

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## DISCUSSION

The chemical characterization of the RASS used in the present study is the most comprehensive characterization of SS in an inhalation study to date. It allows a detailed comparison of RASS to ETS. The most coherent and representative recent publication on ETS composition involved volunteers in an environmental chamber who smoked the 50 top-selling US cigarette brands (Martin et al., 1997). Respirable suspended particles (RSP) as reported in this study can be considered to be solely particulate matter due to the controlled conditions employed, and would thus correspond to the definition of TPM in the present study. On the basis of TPM or RSP, the high RASS concentration in the present study was about 10-fold higher than the extreme ETS concentrations employed in the market cigarette study (Martin et al., 1997) (Figure 6). The ratios of the other SS constituents determined in both studies are in the same order of magnitude suggesting a proportional composition of 1R4F RASS and ETS generated from market cigarettes. In the most thorough recent ETS field study performed in 16 US cities, only those ETS constituents were determined which have generally been used as ETS markers (Jenkins et al., 1996). The comparison of the ETS concentrations of these constituents to those determined in RASS again supports the representative character of RASS for ETS (Figure 6).

The RASS concentrations in the present study were 2 to 3 orders of magnitude above the ETS concentration determined by Jenkins et al. (1996) in smokers' homes, and approximately 2 orders of magnitude above maximum average RSP concentrations contributed by ETS for residential (0.1 µg/l) and office (0.06 µg/l) environments reported by the US EPA (1992). Based on these RSP concentrations and a respiratory minute volume of 7 l/min for a 70 kg person, the daily RSP dose taken up in residences during 16 h and in offices during 8 h can

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be estimated to be approximately 10 and 3 µg/kg, respectively. The daily TPM dose taken up by the rats in the present study was 3 orders of magnitude higher.

As a result of dilution, the concentrations of most RASS components decreased in proportion to the concentrations of either the particulate matter or gas phase markers. The major exceptions to this rule were the low concentrations of nicotine, formaldehyde, and catechol found in the whole-body compared to the head-only exposure chambers. Nicotine is known to adsorb with high affinity to all kinds of surface materials. The same lack of proportional dilution associated with the exposure mode was found for the nicotine concentration in MS (Chen et al. 1989). Formaldehyde is a highly reactive compound which, under whole-body exposure conditions, even reacts with rat excretion products and/or fur (unpublished results; Kewitz and Welsch, 1966). No explanation is available to date for the comparatively low catechol concentrations in the whole-body exposure groups.

Biomonitoring via the analysis of HbCO proportions in blood, hemoglobin adducts of 4-AB, and nicotine metabolites in urine confirmed exposure of the rats as planned. Further, valuable information was obtained on the feasibility of using these biomarkers in smoke inhalation studies and on their concentration/dose-responses.

After establishing a steady-state equilibrium, the blood HbCO proportion is directly proportional to the RASS CO concentration. However, the HbCO proportion does not reflect the overall dose of RASS taken up by the rats, especially when considering the differences in the daily whole-body and head-only exposure durations. The levels of the 4-AB hemoglobin adduct corresponded to the daily TPM doses for all groups. This adduct is considered to reflect the overall dose of metabolically activated 4-AB over the lifetime of the erythrocytes. However, for smoke inhalation studies, another explanation for the increase in this adduct with increasing TPM doses cannot be ruled out: The metabolic activation of 4-AB might be

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dose-dependently induced, similar to the induction seen for benzo(a)pyrene metabolism in previous cigarette smoke inhalation studies (*e.g.*, Gairola, 1987; Haussmann et al., 1998). Indeed, pretreatment of rats with polycyclic aromatic hydrocarbons was shown to shift the hepatic metabolism of 4-AB towards activated metabolites and to proportionally increase the yield of protein adducts (Orzechowski et al., 1994). In any case, even if the increase in the 4-AB hemoglobin adduct seen in the present study is not a specific marker for 4-AB uptake, it might be a marker for the uptake of compounds capable of inducing 4-AB metabolism. The source of 4-AB responsible for the formation of the adduct level found in the sham-exposed group remains to be determined. A similar 4-AB background adduct level was reported by others (Bryant et al., 1987). High background adduct levels may also be responsible for the failure to detect possible increases in hemoglobin adduct levels of other aromatic amines associated with RASS inhalation.

The biomonitoring end points discussed above do not necessarily reflect the route of uptake of the respective compounds. This is especially important for whole-body exposure. Therefore, care has to be taken in determining the dose taken up by inhalation *vs.* the dose taken up by other routes. During whole-body exposure, aerosol is known to deposit on the fur of the rats and its constituents may be taken up either transdermally or orally by grooming (Langard and Nordhagen, 1980; Wolff et al., 1982; Iwasaki et al., 1988). This increases the total dose taken up and may exaggerate the associated toxicity. It should be kept in mind, however, that humans are exposed to ETS in a manner comparable to whole-body exposure. A certain degree of transdermal uptake of ETS constituents can thus be expected; its contribution to the total uptake remains to be determined. In rats, the gastrointestinal uptake of test material by grooming adds to the non-inhalative transdermal uptake, and this is certainly not representative for human ETS exposure. Chen et al. (1995) concluded that whole-body exposure increased the amount of cigarette smoke particles passing from the fur

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into the gastrointestinal tract by about a factor of two compared to nose-only exposure. Mauderly et al. (1989) found nicotine concentrations in the plasma and urine of rats which were 5- to 6-fold higher with whole-body MS exposure than with nose-only exposure, based on equal nicotine concentrations in MS. Their corresponding urine cotinine concentrations were 2.6-fold higher. The latter factor fits well with the 2- to 3-fold higher amounts of nicotine metabolites excreted by whole body vs. head-only exposed rats in the present study, based on equal nicotine inhalation. Thus, for RASS, as is the case for other aerosols, whole-body exposure is associated with a significant uptake of test materials by routes other than inhalation.

The sum of the five nicotine metabolites determined in HO12 accounted for almost 50 % of the calculated inhaled nicotine dose per day. About the same percentage was obtained for the urinary excretion of these five metabolites following intravenous administration of nicotine to male Sprague-Dawley rats (Schepers et al., 1993). This percentage decreased to approximately 20 % in Aroclor 1254-induced Sprague-Dawley rats due to a different metabolic pattern in induced and non-induced rats. These data suggest that the nicotine metabolite pattern of the RASS-exposed rats was similar to that of non-induced rats, although this comparison includes an extrapolation between different rat strains and genders. This interpretation is supported by the lack of any change in the relative pattern of the five metabolites between 3 weeks and 12 months of inhalation.

The above mentioned biomonitoring end points cannot be used to determine the deposition of particulate matter in the lungs. The accumulation of pigmented AM may be an indication of particles accumulating in the lungs following smoke inhalation, since this phenomenon was found in rats following inhalation of whole MS but not in those only exposed to the vapor phase of MS (Davis et al., 1975a and b; Coggins et al., 1980). However, the

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pigmentation may also result from lipid peroxidation or hemoprotein-derived iron incorporation in the AM as a secondary effect due to smoke particle inhalation. The number of AM staining positive for iron/hemosiderin was found to increase in rats following subchronic SS inhalation (CO concentration: 35 ppm; Escolar et al., 1995). In the present study, no iron-specific staining was used, and the slight accumulation of pigmented AM in the high concentration groups was seen in hematoxylin/eosin-stained sections. No such pigmentation was reported for subchronic SS inhalation studies on rats (von Meyerinck et al., 1989; Coggins et al., 1993; Lee et al., 1993; Teredesai and Prühs, 1994; Haussmann et al., 1998). It remains to be investigated whether this pigmentation is due to the higher daily particulate matter dose in the chronic compared to the subchronic studies and/or whether this is related to the chronic inhalation period. AM pigmentation was not seen in the chronic SS/MS inhalation study on A/J mice at 87 µg TPM/l (Witschi et al., 1997a). The discrepancy between the results of these two chronic inhalation studies remains to be resolved in view of a possible use of AM pigmentation as a pulmonary particulate biomonitor. To date, this discrepancy speaks for a species-specific secondary source for the pigmentation rather than for a simple endocytosis of TPM.

A dose-dependent increase in the fluorescence of AM from bronchoalveolar lavage was seen in the present study. This fluorescence has not been described in SS inhalation studies to date. Increased AM fluorescence was reported for MS-exposed rats (Coggins et al., 1980; Sköld et al., 1993) as well as for smokers compared to nonsmokers (Vassar et al., 1960; Sköld et al., 1989). There was no progression of this effect between 6 and 12 months of RASS inhalation, suggesting either a saturation of the effect as discussed for the AM fluorescence in smokers (Sköld et al., 1989) or the constitution of a steady-state equilibrium which would be dependent on the RASS concentration rather than the daily or accumulating dose. The nature of the MS-dependent fluorescence is still open, and similar causes have been discussed as for the histologically observed AM pigmentation (*e.g.*, Sköld et al., 1992).

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A possible relationship between AM pigmentation and fluorescence remains to be investigated.

The degree of body weight gain reduction observed for WB12 can be interpreted as a sign of toxicity and demonstrates that the study design would satisfy the requirements set by the OECD (1981) or others for the highest dose level in a carcinogenicity study.

With few exceptions, the histopathological changes observed in the nose were qualitatively and quantitatively similar to those observed following subchronic RASS inhalation (Haussmann et al., 1998) indicating that there was no progression of these findings. This analogy further suggests that these effects, except for the minimal atrophy in the olfactory epithelium, could be considered adaptive responses to repeated irritation as shown for subchronic studies (von Meyerinck et al., 1989; Coggins et al., 1993; Haussmann et al., 1998). In addition to the reserve cell hyperplasia seen in the anterior part of the nose in the subchronic studies, slight squamous metaplasia of the respiratory epithelium in this part of the nose was seen in WB12. The appearance of this effect in WB12 might be due to the higher daily TPM dose compared to the subchronic studies and/or the chronic inhalation period. Some additional epithelial changes in nose level 2, although not seen in subchronic SS inhalation studies, are not considered relevant due to their very low incidence and degree. The eosinophilic material observed in olfactory epithelial sustentacular cells at levels 2 and 3 has not been reported in cigarette smoke inhalation studies. The significance of this finding is not clear.

In the larynx, the hyperplastic and metaplastic changes were the most pronounced. Those seen in HO12 were in line with those observed in the previous subchronic head-only exposure study (Haussmann et al., 1998) on the basis of an assumed linear concentration-response relationship (Figure 7A). However, the degree of the changes seen in WB6 and WB12

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exceeded those observed in HO12 and also those seen in the previous subchronic head-only exposure studies. Normalizing these larynx effects to daily TPM doses reveals a dose-response relationship with no distinction between subchronic or chronic inhalation periods for the squamous metaplasia (Figure 7B) as well as the hyperplasia. Thus, there seems to be no progression with prolonged inhalation.

An association of most of the histopathological changes with the particulate matter phase of the SS aerosols was assumed in the previous subchronic study (Haussmann et al., 1998). The upper respiratory tract results of the present study support this interpretation and place emphasis on the daily particulate matter dose rather than on concentration (WB12 *vs.* HO12 / WB6). This contrasts with gaseous irritants, such as formaldehyde, which induced epithelial hyperplasia and metaplasia in the anterior part of the nose depending on the concentration rather than on the daily inhaled dose (Wilmer et al., 1989).

The base of epiglottis was the most sensitive site of the respiratory tract in this and the subchronic study (Haussmann et al., 1998). The hyperplastic and metaplastic changes seen there were not seen in SS inhalation studies performed by other laboratories (von Meyerinck et al., 1989; Coggins et al., 1993).

The only relevant histopathological finding in the lower respiratory tract following chronic RASS inhalation was slight reserve cell hyperplasia in the bronchial respiratory epithelium, which was not seen in the subchronic SS inhalation studies (von Meyerinck et al., 1989; Coggins et al., 1993; Teredesai and Prühs, 1994; Haussmann et al., 1998). It remains to be investigated whether this effect is due to the chronic inhalation period and/or the higher concentration or dose in the present study compared to the previous ones. Morphometric analyses to assess the pulmonary changes as reported by Escolar et al. (1995) were not performed in the present study.

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The composition of the bronchoalveolar lavage cells did not indicate an inflammatory response to chronic RASS inhalation, although there was a slight but statistically insignificant increase in the number of AM, a response similar to that seen in rats following MS inhalation (Gairola, 1986; Bjermer et al., 1993; Miller et al., 1996). Also for MS, Mauderly et al. (1989) reported an increase in the concentration of PMNL in the bronchoalveolar lavage fluid of rats, whereas no statistically significant responses were found by others for this species (Gairola, 1986; Bjermer et al., 1993; Miller et al., 1996). Mice were considered to be more sensitive to MS inhalation with regard to PMNL responses (Gairola, 1986). However, no histopathological indication of an inflammatory response was seen by Witschi et al. (1997a) when A/J mice were chronically exposed to a mixture of SS and MS at a TPM concentration as high as 87 µg/l. On the other hand, pronounced effects on rat bronchoalveolar lavage cell composition indicating a strong inflammatory response were found following inhalation of aerosols including insoluble particles, such as Diesel engine exhaust, at particulate matter concentrations far below those used in cigarette smoke inhalation studies (Henderson et al., 1988). Pulmonary inflammation following particle inhalation has been associated with lung carcinogenesis in experimental animals (Dungworth et al., 1994).

An increase in the formation of 8-OHdG has been investigated in the present study as a marker for oxidative stress since this end point has been associated with carcinogenesis (Floyd, 1990) and with cigarette smoking (Asami et al., 1996, 1997). The observed increase of the 8-OHdG levels in the nasal epithelia at the highest RASS concentration was only in part statistically significant and consistent following 6 or 12 months of inhalation. The biological relevance of this effect remains to be investigated. With regard to its relation to carcinogenesis, it is interesting to note that Witschi et al. (1997a,b) have observed increased cell proliferation and epithelial lesions in the nose but did not specifically report nasal tumors

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using SS/MS concentrations up to one order of magnitude higher than those in the present study.

In contrast to the results obtained for the nasal epithelia, in the lungs no statistically significant effect in the levels of 8-OHdG was found following 6 and 12 months of RASS inhalation; indeed, the levels tended to decrease. This might be plausible in view of several adaptive antioxidant responses reported for lungs following cigarette smoke exposure, *e.g.*, the accumulation of vitamins E and C or increased activities of glutathione peroxidase or superoxide dismutase, as reviewed by Chow (1993). Furthermore, the repair of oxidative DNA modifications might be induced as a consequence of chronic exposure as reported for the repair activity in the leukocytes of smokers (Asami et al., 1996). However, the above results do not preclude possible oxidative changes in particular pulmonary cell types not detectable by analyzing the homogenate of the whole organ. At the dose level used in the present study and within the limitations of the method, these data do not support the hypothesis of a free radical-mediated oxidative stress in the lungs of SS-exposed rodents (Witschi et al., 1997b).

The determination of the urinary excretion of 8-OHdG enables an overall assessment of oxidative DNA modifications and repair in the whole body (Shigenaga et al., 1990). A 50 % increase of the 8-OHdG excretion was reported for smokers (Loft et al., 1992). In the present study, a dose dependent increase of 8-OHdG excretion in the whole-body exposed rats was seen following 5 months of RASS inhalation. After 12 months, however, this effect was no longer seen. It is not known whether this can be explained by adaptive processes in the course of chronic RASS inhalation. Further, a distinction between effects related to the head-only RASS exposure and those related to the oxidative stress possibly associated with a certain degree of immobilization (Liu et al., 1996) imposed by the head-only exposure mode is not possible. Another point to consider is the higher catechol concentration in HO12

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compared to WB12: Aqueous catechol solutions were capable of generating 8-OHdG in vitro (Leanderson and Tagesson, 1990), and a combined intraperitoneal administration of catechol and hydroquinone or phenol resulted in an increased level of 8-OHdG in mouse bone marrow (Kolachana et al., 1993).

Using the whole-body exposure mode, the rats could be exposed for a daily duration longer than that considered feasible for head-only exposure, thereby maximizing the daily RASS dose. This also enabled evaluations on the impact of the daily RASS dose *vs.* the RASS concentration as demonstrated, *e.g.*, for the histopathological changes in the larynx. For most of the end points investigated in this study, the exposure mode *per se* did not influence the RASS-induced changes, which were mainly local effects in the respiratory tract. It did, however, impact the evaluation of the body weight gain, which was essentially the only sign of systemic toxicity in the present study. In addition, the exposure mode might have also influenced the formation and excretion of 8-OHdG. For cigarette smoke carcinogenicity studies, whole-body exposure must be considered to be a substantial confounding factor: (1) The composition of the cigarette smoke aerosol, either RASS (present study) or MS (Chen et al., 1989), was found to artificially change in the whole-body situation. (2) There is a considerable non-inhalative uptake of smoke components which, based on the nicotine biomonitoring data for RASS (present study) and MS (Mauderly et al., 1989; Chen et al., 1995), might exceed the inhalative uptake by far. NNK, a compound structurally similar to nicotine, is a rodent lung carcinogen regardless of the route of administration, including dermal application (LaVoie et al., 1987), with a preferential formation of adenomas and adenocarcinomas (Hoffmann et al., 1996). Furthermore, pulmonary DNA adducts were found after topical application of MS condensate (Randerath et al., 1988) or Diesel engine exhaust extracts (Gallagher et al., 1990) to mouse skin. The extent of non-inhalative uptake might be different for nicotine and other compounds, since no influence of the exposure mode was

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seen on the levels of the 4-AB hemoglobin adduct (RASS, this study) or those of pulmonary DNA adducts (MS, Mauderly et al., 1989) detected by the postlabeling method.

The role of particulate matter-related smoke constituents in the pulmonary carcinogenicity in A/J mice has recently been questioned by Witschi et al. (1997b), who reported that the carcinogenic potential of a HEPA-filtered mixture of SS and MS would be similar to that of the unfiltered smoke. However, the susceptibility as well as the relevance of this animal model remains to be clarified (Maronpot et al., 1986), especially at the dose/toxicity level employed.

In summary, for histopathological changes, pulmonary inflammation, or oxidative DNA damage, there was little indication of progression or occurrence of new effects following an extended inhalation period of 12 months. The two slight histopathological changes which were not seen in the subchronic studies, *i.e.*, squamous metaplasia in the anterior nasal respiratory epithelium and the bronchial reserve cell hyperplasia, might well be due to the higher concentration and daily dose in the chronic compared to the subchronic studies. However, it cannot be ruled out that these effects are due to the chronic inhalation period. Chronic head-only exposure to cigarette smoke was shown to be technically feasible and is considered preferable to whole-body exposure in order to avoid artificial changes in smoke composition and the considerable non-inhalative uptake of smoke constituents.

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**TABLE 1.** Experimental Groups

| Group Code | TPM Target Concentration (µg/l) | Exposure Mode, Daily Exposure Duration | Daily TPM Target Dose (µg/l x h) | Total Number of Rats Initially Exposed |
|---|---|---|---|---|
| WB0 | 0 | whole-body, 12 h | 0 | 96 |
| WB6 | 6 | " | 72 | 48 |
| WB12 | 12 | " | 144 | 96 |
| HO12 | 12 | head-only, 7 h | 84 | 48 |

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**TABLE 2.** Concentrations of Selected Smoke Components in the Test Atmospheres

| Parameter | Unit of Measure | n | Exposure Groups WB0 | WB6 | WB12 | HO12 |
|---|---|---|---|---|---|---|
| TPM | µg/l | 257 to 259 | n.d. | 5.9 ± 0.5 | 11.9 ± 0.6 | 12.1 ± 0.7 |
| carbon monoxide | ppm | 259 | n.d. | 27 ± 2 | 51 ± 2 | 42 ± 2 |
| carbon dioxide (chamber inlet) | " | 11 to 12 | 270 ± 130 | 390 ± 150 | 510 ± 160 | 480 ± 150 |
| " (chamber outlet) | " | 11 to 12 | 1060 ± 90 | 1400 ± 240 | 1410 ± 260 | 2000 ± 650 |
| nicotine | µg/l | 56 to 60 | n.d. | 0.94 ± 0.38 | 1.95 ± 0.57 | 2.35 ± 0.56 |
| 3-ethenyl pyridine | " | 52 to 53 | - | 0.20 ± 0.05 | 0.42 ± 0.06 | 0.37 ± 0.05 |
| ammonia | " | 11 | 1.3 ± 0.7 | 2.8 [a] | 5.2 [b] | 4.5 ± 1.2 |
| solanesol | " | 13 | - | 0.06 ± 0.01 | 0.12 ± 0.02 | 0.10 ± 0.02 |
| formaldehyde | ppm | 12 | - | 0.10 ± 0.03 | 0.17 ± 0.04 | 0.50 ± 0.07 |
| acetaldehyde | " | 12 | - | 0.67 ± 0.05 | 1.31 ± 0.06 | 1.11 ± 0.05 |
| acrolein | " | 12 | - | 0.13 ± 0.01 | 0.24 ± 0.01 | 0.22 ± 0.01 |
| nitric oxide | " | 11 | 0.01 [c] | 0.84 ± 0.08 | 1.68 ± 0.09 | 1.42 ± 0.13 |
| nitric oxides | " | 11 | 0.03 ± 0.05 | 0.87 ± 0.10 | 1.73 ± 0.10 | 1.47 ± 0.13 |
| 1,3-butadiene | µg/l | 5 to 6 | - | 0.07 ± 0.03 | 0.14 ± 0.06 | 0.13 ± 0.07 |
| isoprene | " | 5 to 6 | - | 1.1 ± 0.2 | 2.5 ± 0.6 | 2.1 ± 0.7 |
| benzene | " | 5 to 6 | - | 0.16 ± 0.02 | 0.33 ± 0.05 | 0.29 ± 0.04 |
| toluene | " | 5 to 6 | - | 0.47 ± 0.05 | 0.90 ± 0.15 | 0.73 ± 0.10 |

[a] median; quartiles: 2.3 and 4.0 µg/l
[b] median; quartiles: 4.7 and 6.2 µg/l
[c] median; quartiles: 0.00 and 0.03 ppm

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**TABLE 2.** Concentrations of Selected Smoke Components in the Test Atmospheres (cont.)

| Parameter | Unit of Measure | n | Exposure Groups | | | |
|---|---|---|---|---|---|---|
| | | | WB0 | WB6 | WB12 | HO12 |
| phenols | | | | | | |
| phenol | ng/l | 4 | - | 2.8 ± 0.5 | 6.9 ± 0.7 | 6.6 ± 0.9 |
| o-cresol | " | 4 | - | 0.48 ± 0.06 | 1.19 ± 0.10 | 1.12 ± 0.13 |
| m-cresol | " | 4 | - | 0.53 ± 0.08 | 1.32 ± 0.14 | 1.37 ± 0.17 |
| p-cresol | " | 4 | - | 1.1 ± 0.2 | 2.7 ± 0.3 | 2.6 ± 0.3 |
| catechol | " | 4 | - | 7.4 ± 2.2 | 16.7 ± 4.1 | 29.7 ± 4.9 |
| resorcinol | " | 4 | - | 0.24 ± 0.02 | 0.44 ± 0.04 | 0.43 ± 0.05 |
| hydroquinone | " | 4 | - | 32 ± 5 | 69 ± 8 | 71 ± 7 |
| polycyclic aromatic *hydrocarbons* | | | | | | |
| fluoranthene | pg/l | 2 | - | 170/120 | 370/270 | 510/440 |
| pyrene | " | 2 | - | 160/120 | 320/260 | 430/370 |
| benzo(a)anthracene | " | 2 | - | 140/ 90 | 260/190 | 270/210 |
| chrysene | " | 2 | - | 320/220 | 610/490 | 560/510 |
| benzo(-)fluoranthene | " | 2 | - | 100/ 80 | 200/170 | 170/160 |
| benzo(a)pyrene | " | 2 | - | 65/ 55 | 132/123 | 112/121 |
| indeno(1,2,3,-cd)pyrene | " | 2 | - | 25/ 28 | 53/ 53 | 42/ 50 |
| dibenzo(-)anthracenes | " | 2 | - | 6/ 7 | 14/ 14 | 11/ 11 |
| benzo(ghi)perylene | " | 2 | - | 16/ 19 | 39/ 37 | 30/ 33 |

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**TABLE 2.** Concentrations of Selected Smoke Components in the Test Atmospheres (cont.)

| Parameter | Unit of Measure | n | Exposure Groups | | | |
|---|---|---|---|---|---|---|
| | | | WB0 | WB6 | WB12 | HO12 |
| N-nitrosamines | | | | | | |
| N-nitrosodimethylamine | ng/l | 2 | n.d. | 0.30/n.d. | 0.58/n.d. | 0.57/n.d. |
| N-nitrosopyrrolidine | " | 2 | n.d. | n.d. | n.d. | 0.14/0.12 |
| N-nitrosonornicotine | " | 2 | n.d. | n.d. | n.d. | 0.21/0.25 |
| N-nitrosoanatabine | " | 2 | n.d. | n.d. | n.d. | n.d. |
| N-nitrosoanabasine | " | 2 | n.d. | n.d. | n.d. | n.d. |
| NNK | " | 2 | n.d. | 1.26/1.12 | 2.08/1.98 | 2.33/2.17 |
| cadmium | pg/l | 2 | n.d. | 280/310 | 470/590 | 460/520 |

*Note.* - Data are given as mean ± standard deviation (except when n = 2).
- WB0: whole-body sham-exposed control group;
- WB6 and WB12: whole-body exposure groups at 6 and 12 µg TPM/l, respectively;
- HO12: head-only exposure group at 12 µg TPM/l.
- "-": not determined.
- n.d.: not detectable.
- Detection limits: TPM: 0.1 µg/l, CO: 1.5 ppm, nicotine: 0.05 µg/l, N-nitrosodimethylamine: 0.16 ng/l, N-nitrosopyrrolidine: 0.11 ng/l, N-nitrosonornicotine: 0.21 ng/l, N-nitrosoanatabine and N-nitrosoanabasine: 0.19 ng/l, NNK: 0.36 ng/l, cadmium: 10 pg/l.
- Chromium, nickel, lead, and zinc: no RASS-dependent increase above blind filter contents of these metals.

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**TABLE 3.** Histopathological Findings Following 12 Months of RASS Inhalation

| Parameter | Exposure Groups | | | |
|---|---|---|---|---|
| | WB0 | WB6 | WB12 | HO12 |
| nasal cavity, level 1 | | | | |
| respiratory epithelium | | | | |
| reserve cell hyperplasia | 0<br>0/8 | 0.8 ± 0.2 *<br>6/8 | 1.9 ± 0.1 *<br>8/8 | 1.6 ± 0.3<br>7/8 |
| squamous metaplasia | 0<br>0/8 | 0.1 ± 0.1<br>1/8 | 1.0 ± 0.3 *<br>5/8 | 0.3 ± 0.2<br>2/8 |
| goblet cell hyperplasia | 0<br>0/8 | 0<br>0/8 | 0.4 ± 0.4<br>1/8 | 0.1 ± 0.1<br>1/8 |
| nasal cavity, level 2 | | | | |
| respiratory epithelium | | | | |
| reserve cell hyperplasia | 0<br>0/8 | 0.1 ± 0.1<br>1/8 | 0.1 ± 0.1<br>1/8 | 0<br>0/8 |
| olfactory epithelium | | | | |
| reserve cell hyperplasia | 0<br>0/8 | 0<br>0/8 | 0.1 ± 0.1<br>1/8 | 0<br>0/8 |
| atrophy | 0<br>0/8 | 0<br>0/8 | 0.3 ± 0.2<br>2/8 | 0.3 ± 0.2<br>2/8 |
| eosinophilic globules | 0.1 ± 0.1<br>1/8 | 1.1 ± 0.5<br>3/8 | 1.0 ± 0.5<br>4/8 | 1.3 ± 0.5<br>4/8 |
| nasal cavity, level 3 | | | | |
| olfactory epithelium | | | | |
| eosinophilic globules | 0<br>0/8 | 0.6 ± 0.4<br>2/8 | 0.9 ± 0.5<br>3/8 | 1.5 ± 0.6<br>4/8 |
| larynx | | | | |
| base of epiglottis | | | | |
| squamous metaplasia of pseudostratified epithelium | 0<br>0/1 | 2.8 ± 0.3<br>4/4 | 4.0 ± 0.0<br>3/3 | 2.6 ± 0.2<br>5/5 |
| hyperplasia of squamous epithelium | 0<br>0/1 | 3.3 ± 0.3<br>4/4 | 4.0 ± 0.0<br>3/3 | 2.8 ± 0.2<br>5/5 |

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**TABLE 3.** Histopathological Findings Following 12 Months of RASS Inhalation (cont.)

| Parameter | Exposure Groups | | | |
|---|---|---|---|---|
| | WB0 | WB6 | WB12 | HO12 |
| trachea | | | | |
| bifurcation | | | | |
| reserve cell hyperplasia of respiratory epithelium | 0<br>0/7 | 0<br>0/7 | 0.4 ± 0.2<br>3/7 | 0.1 ± 0.1<br>1/7 |
| lungs | | | | |
| bronchi, respiratory epithelium | | | | |
| reserve cell hyperplasia | 0<br>0/8 | 0.6 ± 0.3 *<br>4/8 | 0.9 ± 0.3 *<br>5/8 | 0.9 ± 0.1<br>7/8 |
| goblet cell hyperplasia | 1.1 ± 0.5<br>4/8 | 0.3 ± 0.2<br>2/8 | 1.1 ± 0.5<br>4/8 | 0.9 ± 0.5<br>3/8 |
| alveoli | | | | |
| accumulation of pigmented macrophages | 0<br>0/8 | 0<br>0/8 | 0.6 ± 0.4<br>3/8 | 0.9 ± 0.1<br>7/8 |

*Note.* Histopathological findings are given as mean score ± SE and incidence. Due to the sectioning problems, only few larynges per group could be evaluated, thereby precluding statistical analysis.

* For whole-body exposed groups: statistically significantly different from WB0.

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**TABLE 4.** Bronchoalveolar Lavage Cell Composition Following 12 Months of RASS Inhalation

| | Exposure Groups | | | |
|---|---|---|---|---|
| Cell Type | WB0 | WB6 | WB12 | HO12 |
| AM | 5.3 ± 0.5 | 5.6 ± 0.6 | 6.4 ± 0.4 | 6.4 ± 0.6 |
| lymphocytes | 0.074 ± 0.009 | 0.100 ± 0.013 | 0.079 ± 0.006 | 0.136 ± 0.019 |
| PMNL | 0.075 ± 0.017 | 0.048 ± 0.014 | 0.036 ± 0.007 | 0.040 ± 0.014 |

*Note.* Bronchoalveolar lavage cells are given in absolute numbers ($10^6$), mean ± SE (no statistically significant differences).

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**Table 5.** Alveolar macrophage fluorescence (relative units).

| | Exposure Groups | | | |
|---|---|---|---|---|
| Inhalation Period | WB0 | WB6 | WB12 | HO12 |
| 6 months | 100 ± 5 | - | 315 ± 14* | - |
| 12 months | 100 ± 5 | 211 ± 19* | 271 ± 15* | 260 ± 16 |

Note: Results are expressed as percentages of WB0 (means ± SE). *For whole-body exposed groups: statistically significantly different from WB0."

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## FIGURE LEGENDS

**Figure 1** Biomonitoring of RASS exposure: A: Blood carboxyhemoglobin determined at three time points (means ± SE), B: Hemoglobin adduct of 4-aminobiphenyl after 12 months of RASS inhalation; daily TPM dose calculated from the respiratory minute volume averaged over the duration of the inhalation period (means ± SE; *: for whole-body exposed groups: statistically significantly different from WB0).

**Figure 2** Biomonitoring nicotine uptake: A: sum of five nicotine metabolites (nicotine-N'-oxide, nornicotine, cotinine, trans-3'-hydroxycotinine, norcotinine) excreted in 24-h urine in month 5 of the inhalation period (means ± SE), B: Data from A, normalized to the inhaled nicotine dose (based on the nicotine concentration in the test atmospheres, the respiratory minute volume calculated according to Guyton (1947), and the body weight of the rats; means ± SE).

**Figure 3** Body weight development (means).

**Figure 4** Tissue levels of 8-hydroxy-deoxyguanosine: A: nasal respiratory epithelium, B: nasal olfactory epithelium, C: lungs (means ± SE; *: for whole-body exposed groups: statistically significantly different from WB0).

**Figure 5** Urinary excretion of 8-hydroxy-deoxyguanosine (means ± SE; *: for whole-body exposed groups: statistically significantly different from WB0).

**Figure 6** Comparison of RASS in present study (HO12) with ETS either generated at an extreme concentration under experimental conditions (Martin et al., 1997) or as determined in a large field study (Jenkins et al., 1996) (abbreviations: CO: carbon monoxide, TPM: total particulate matter, AMM: ammonia, NIC: nicotine, ISO: isoprene, AA: acetaldehyde, NO, nitric

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oxide, TOL: toluene, FA: formaldehyde, EP: 3-ethenyl-pyridine, BEN: benzene, BUT: 1,3 butadiene, SOL: solanesol, CAT: catechol).

**Figure 7** Squamous metaplasia of the pseudostratified epithelium in the larynx, base of epiglottis, in the present and the previous subchronic inhalation study (Haussmann et al., in press) (mean scores ± SE): A: Dependence on the TPM concentration, B: Dependence on the daily TPM dose calculated from the respiratory minute volume averaged over the duration of the inhalation period (n = 4 to 10 rats/group).

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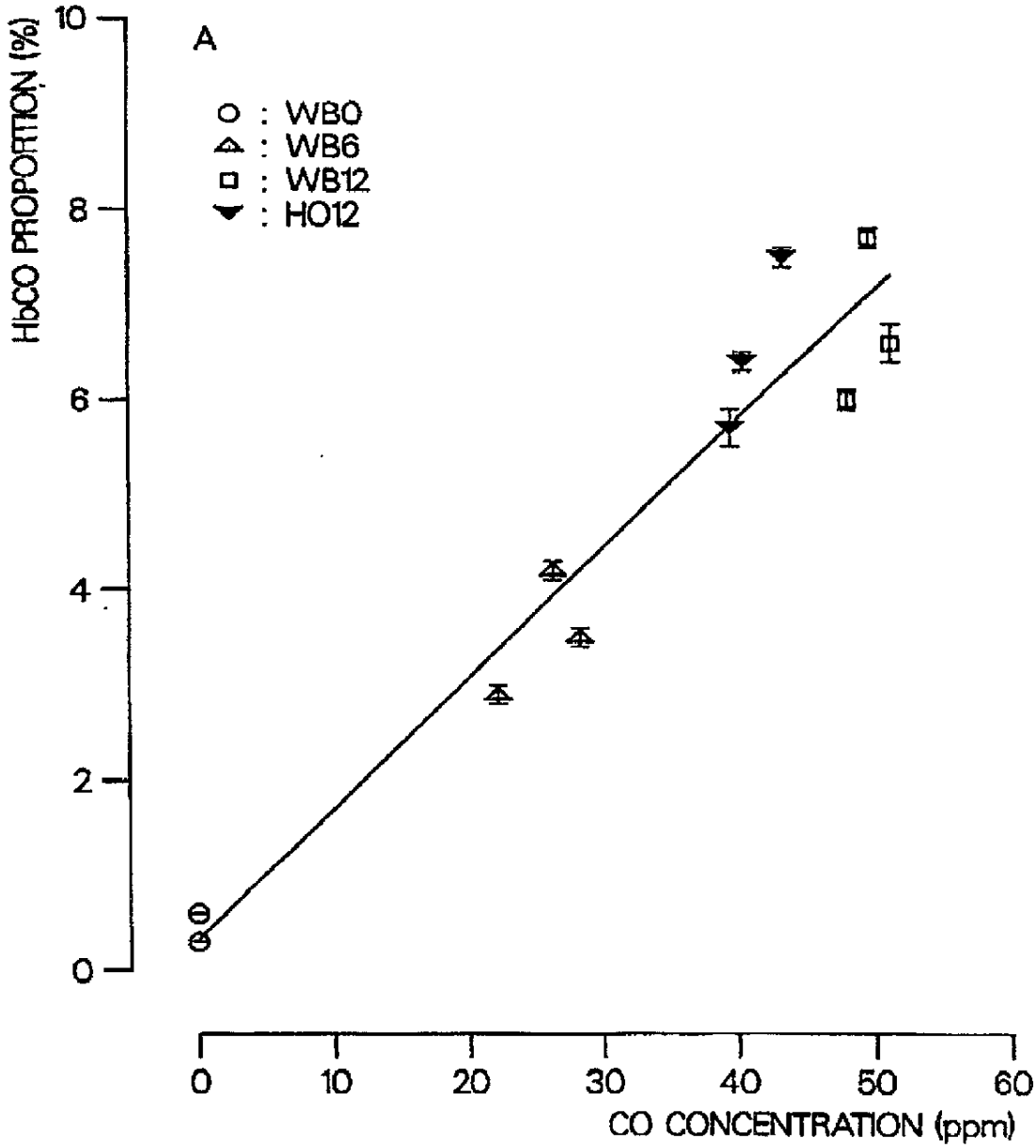


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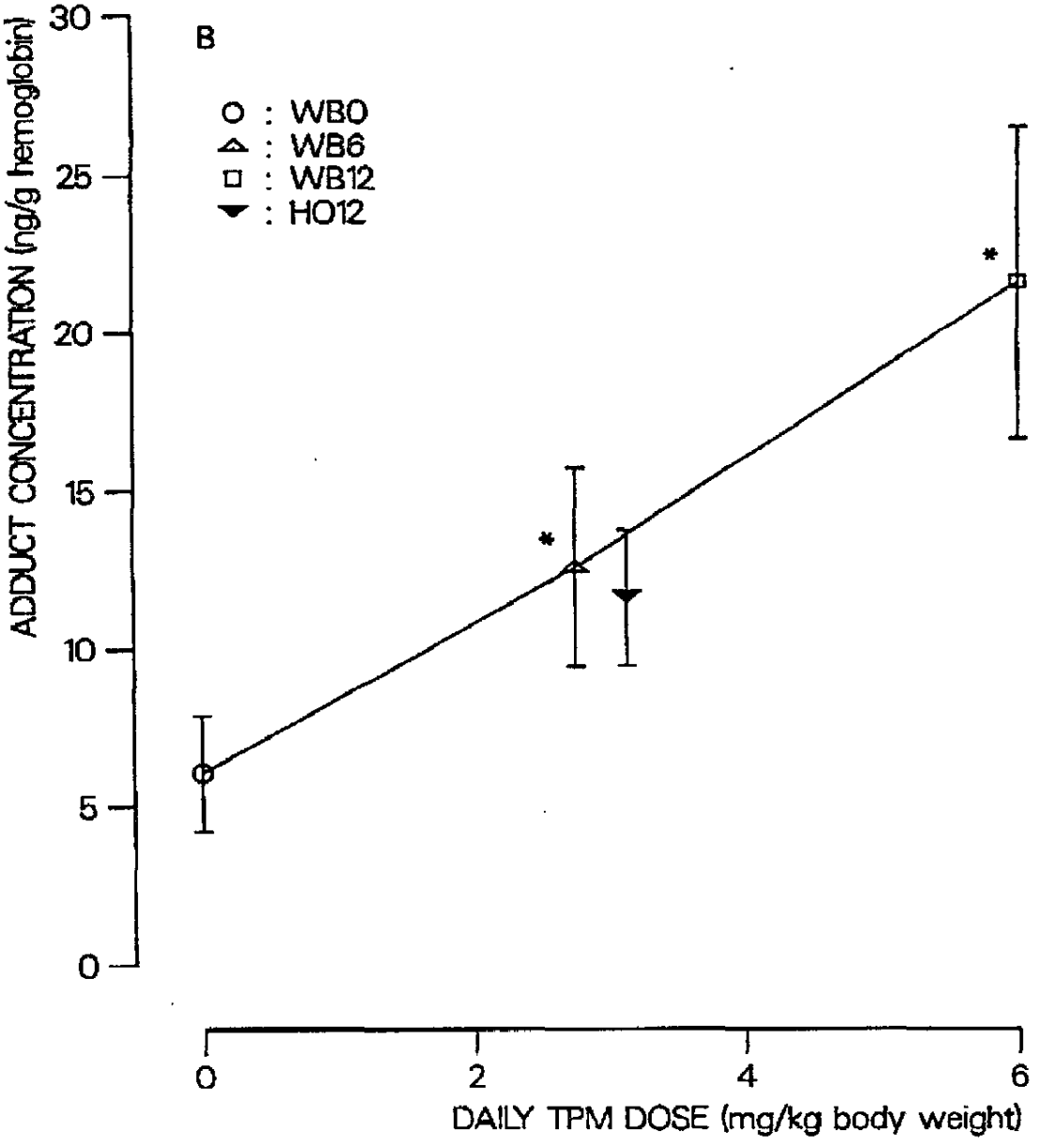


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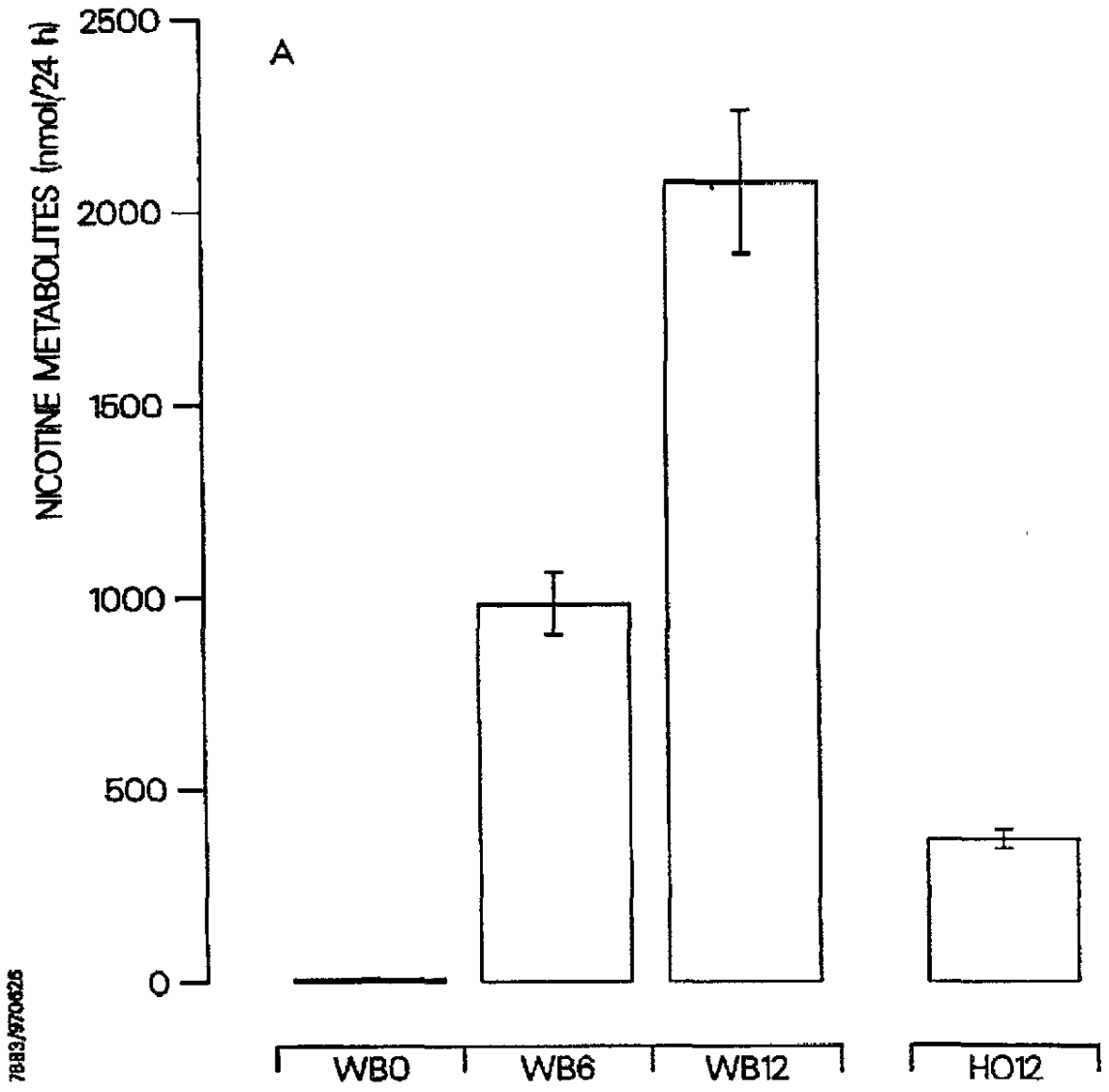


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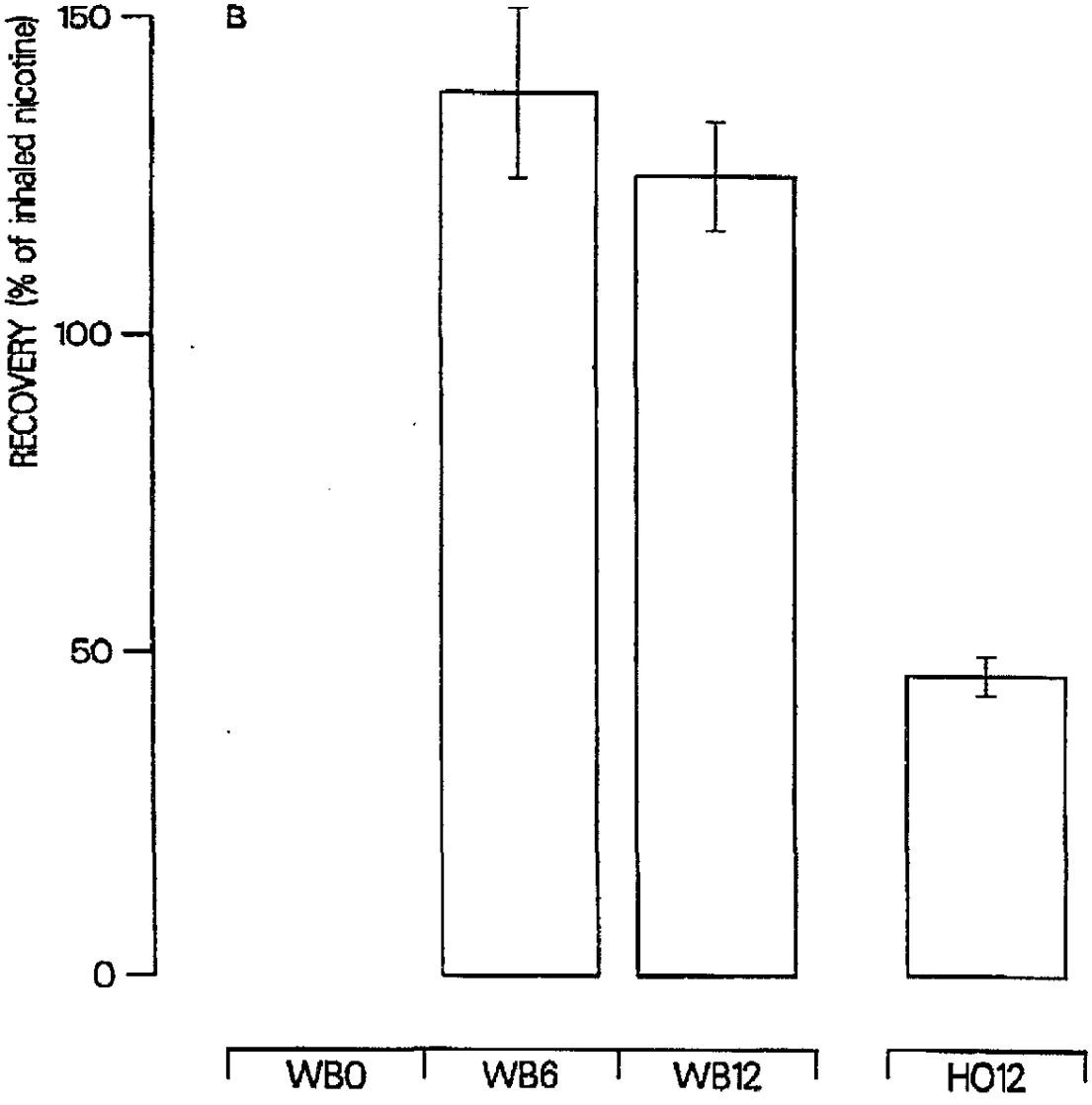


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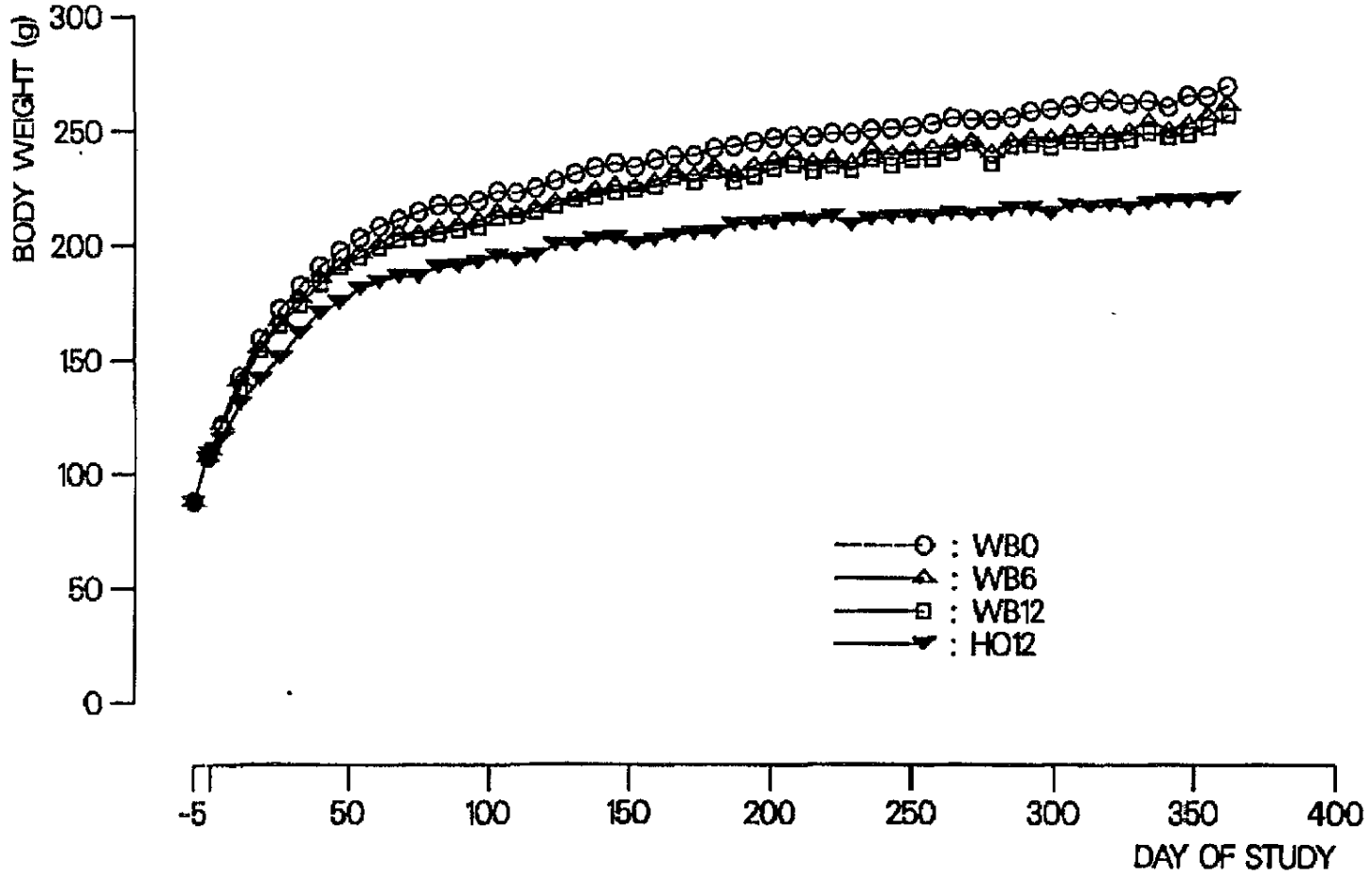


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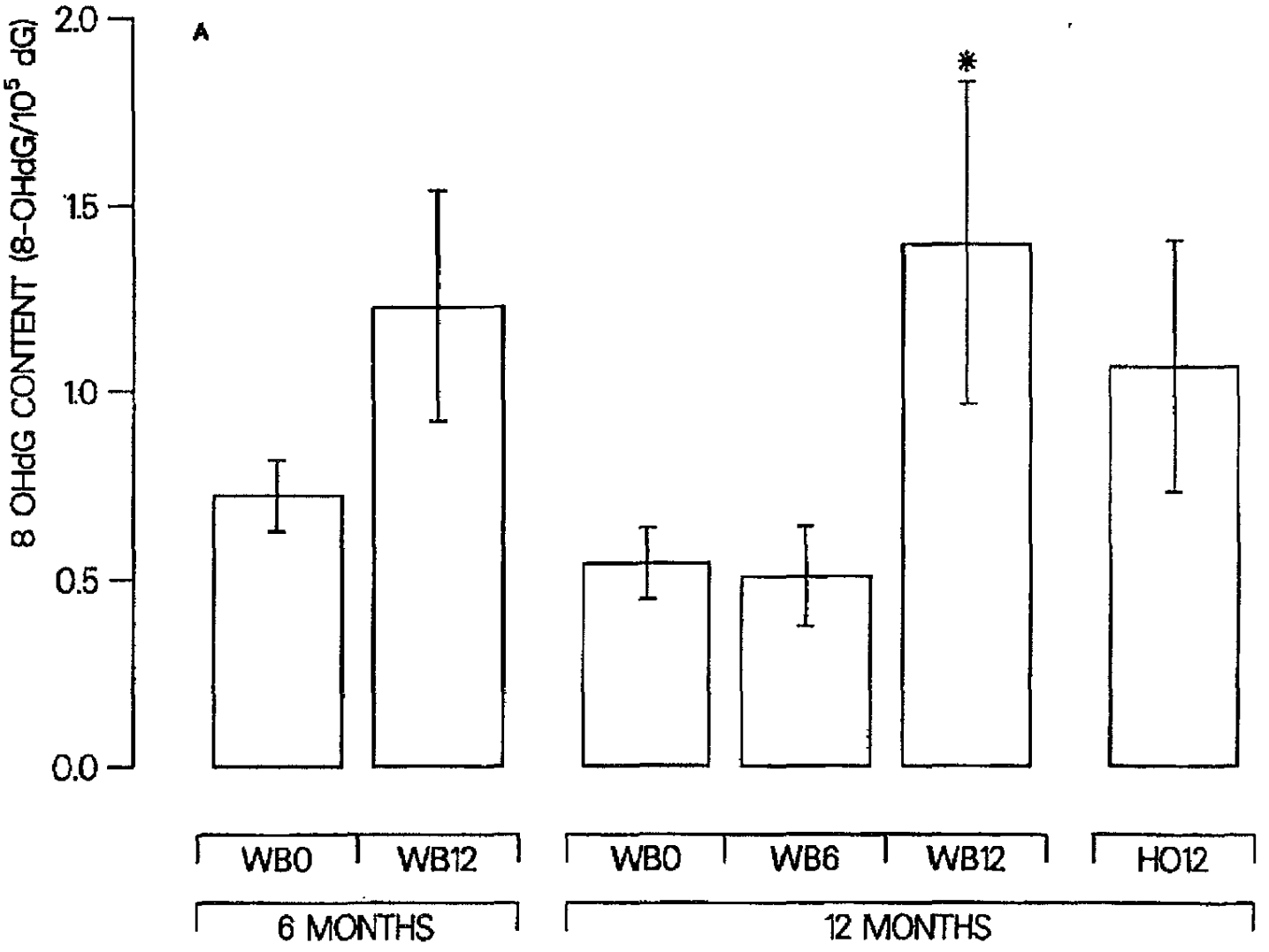


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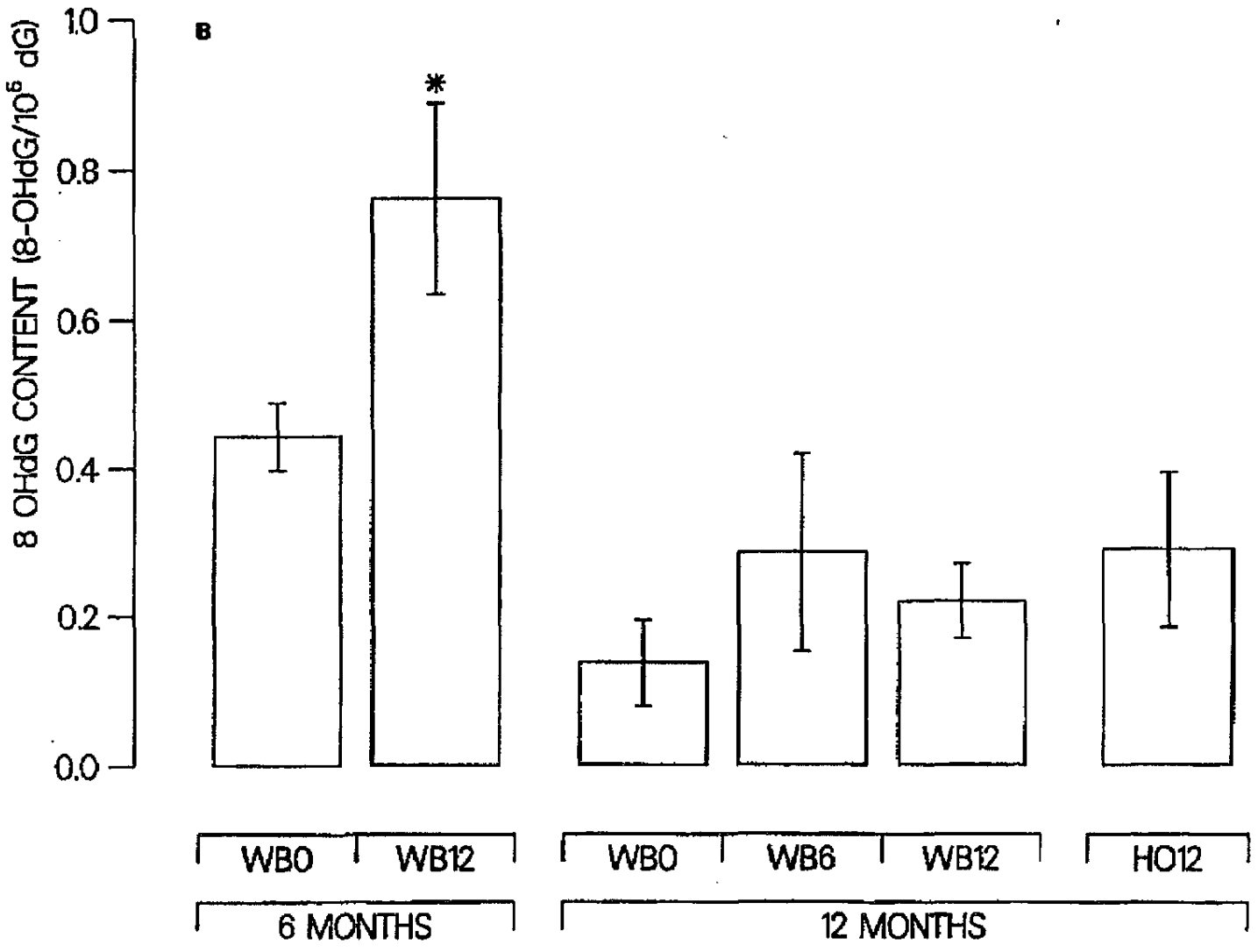


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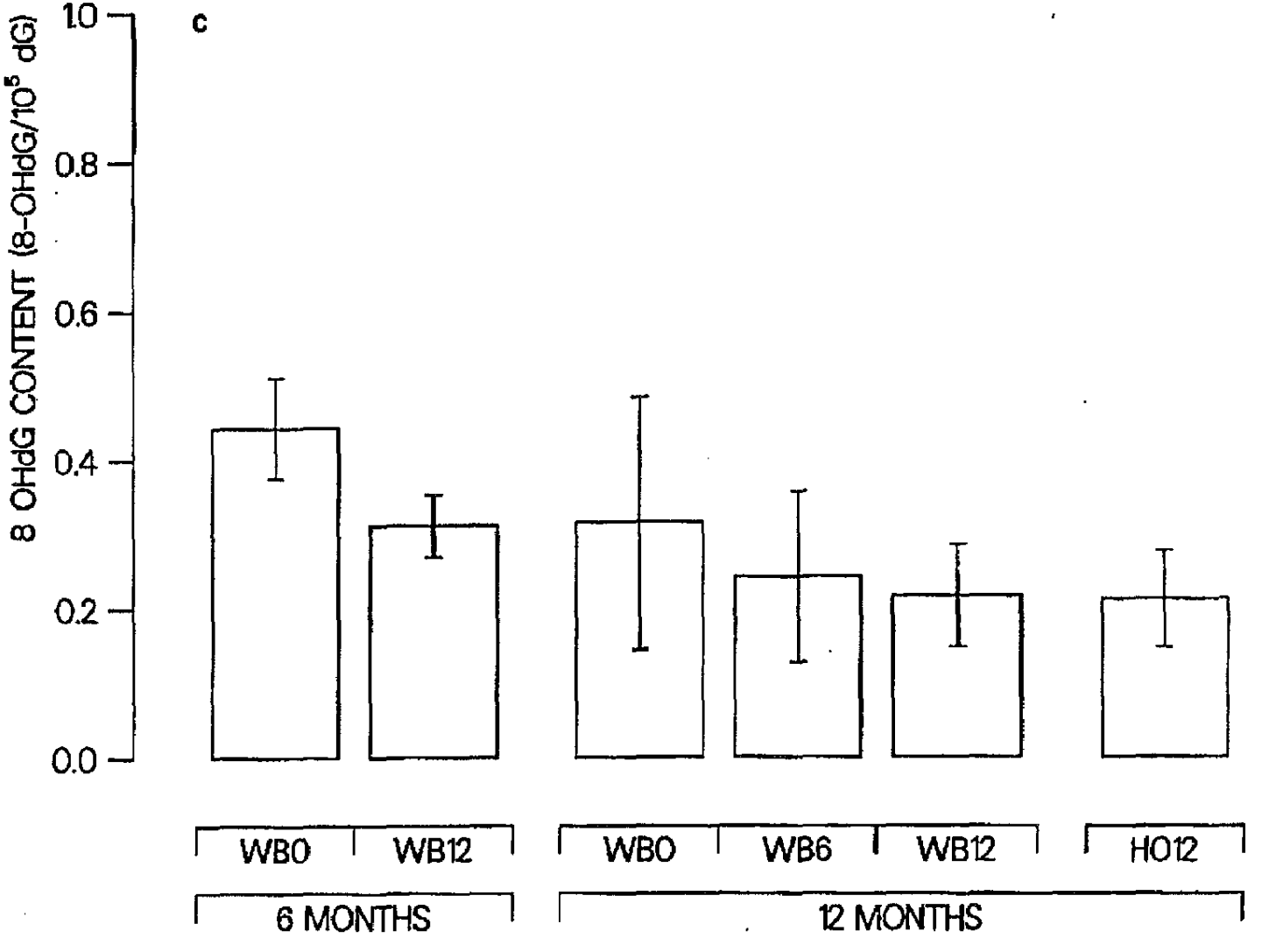


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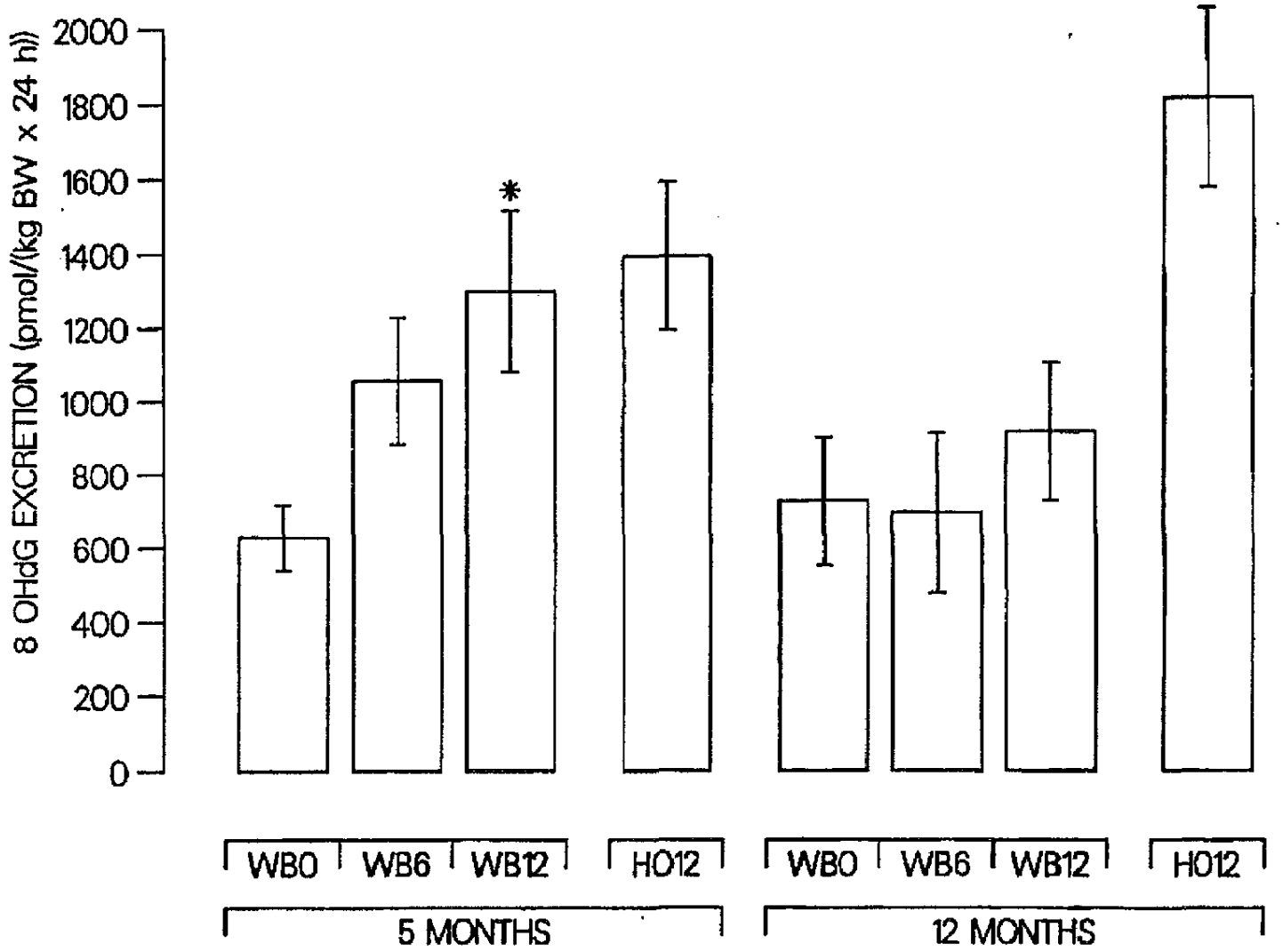


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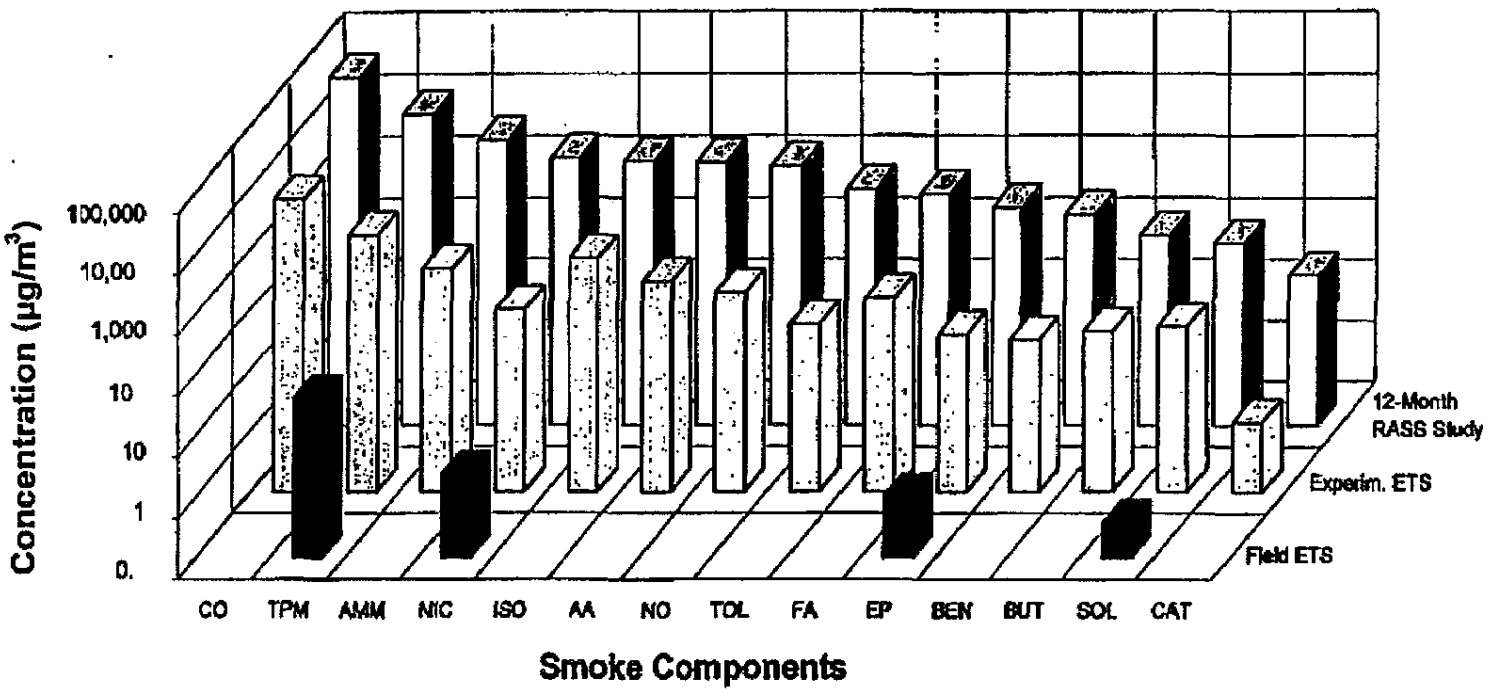


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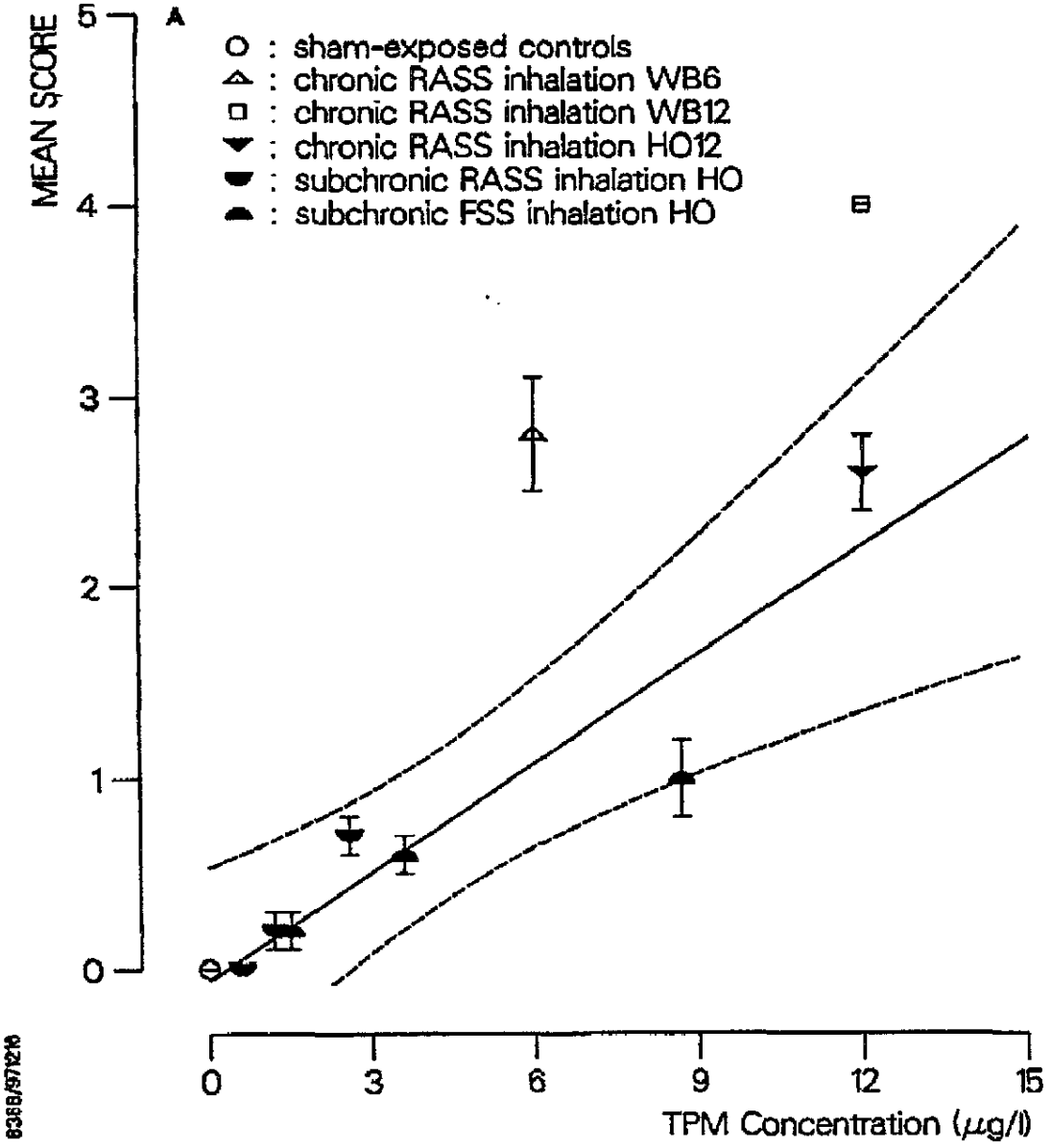


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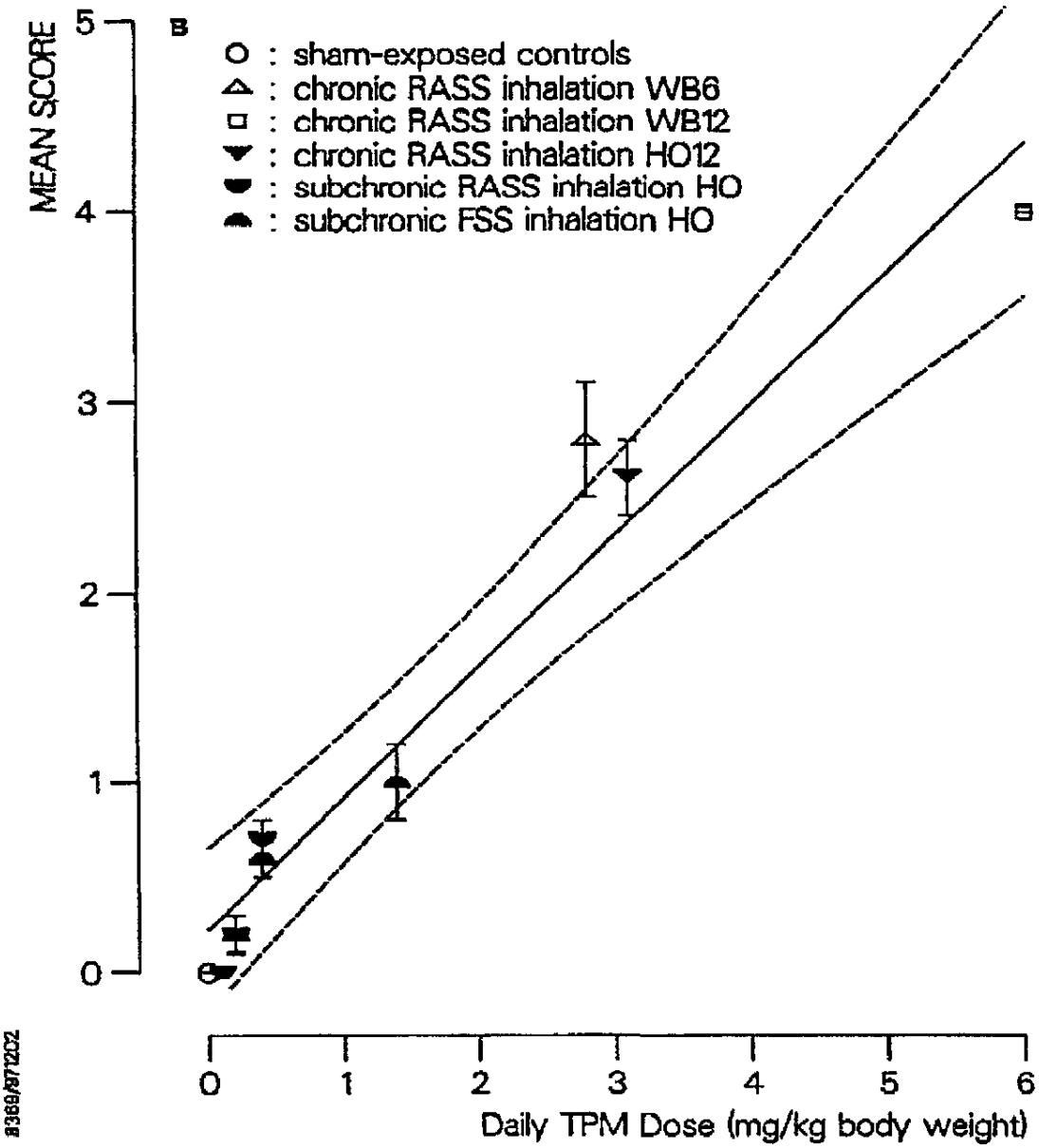


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